# A QUANTITATIVE BIOLOGICAL ASSAY OF VITAMIN K

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The interest in the fat-soluble antihemorrhagic factor or group of factors has grown rapidly during the past years and various methods have become available for the biological assay of the blood clotting agent, vitamin K. The Danish investigators Schønheyder, Dam et al. used a curative blood clotting method which was originally described by Schønheyder ('36, I, II) and has since been subjected to a thorough revision by Dam and Glavind ('38, I). This method of assay is based upon the observation that "animals suffering from vitamin K-deficiency become normal with reference to clotting time in 3 days when given sufficient food containing vitamin K." The California investigators Almquist and Stokstad ('37) described a preventive method which was later slightly modified by Klose et al. ('38); it is "based upon the occurrence of hemorrhage and upon simple measurement of blood clotting time."

It is interesting to note that the Danish method was criticized by the California workers (Almquist, '37) and that the California method appeared open to criticism to the Danish workers (Dam and Lewis, '37). Furthermore, the authors themselves have found fault with their own methods; Dam and Lewis ('37) observed a phenomenon resembling the refection as noted in vitamin B experiments; Dam and Glavind ('38, I) stated that, in spite of the precautions taken, they encountered animals with practically normal coagulation after months on the basal diet; and Almquist and Stokstad ('36) frequently noticed cases of spontaneous recovery. The method

[1] With the technical assistance of J. J. Savage and J. Spataro.

of the Danish workers was found to be difficult and time-consuming by Dann ('38), who adopted the preventive method of the California workers. Dann stated that this technic is "sufficiently accurate for comparative purposes and for a preliminary survey," in spite of the fact that she observed only a relatively small percentage of vitamin K-deficient chicks among her negative controls. Thayer and co-workers ('38) developed "a curative method of assay which appears to have certain advantages over methods previously used (Dam and Schønheyder; Almquist and Stokstad)"; chicks reared on a vitamin K-free diet were used; after marked deficiency had developed, the animals were given an active extract of the vitamin on 3 successive days and on the fourth day blood was drawn to determine the clotting time. Although seemingly valuable data have already appeared in the literature, Smith et al. ('38) believe that a critical survey of the chick assay technic should be made, "for obviously the dosage employed in both man and animals should be based upon accurate assay of the vitamin concentrates employed."

When the vitamin K investigations were started in this Institute, we first employed the curative technic of the Danish investigators. We soon found that a 3-day test period is unduly long, because the blood clotting power of the vitamin K-deficient chick can be rendered normal within a very few hours. This observation (Ansbacher, '38) served as a basis for the quantitative biological assay of vitamin K reported herein.

## EXPERIMENTAL

The experimental animals employed were day-old single comb White Leghorn or White Rock chicks. They were housed in electrically heated brooders equipped with raised wire screen (2 mesh to the inch) bottoms; these brooders were kept in air conditioned rooms with a temperature of about 28°C. Tap water and the basal diets were fed ad libitum.

The basal rations used for the vitamin K assays are described in table 1. The basal protein in diet K-1 was fish meal which had been submitted to continuous extraction with ether

for 7 days. The yeast used in the rations K-1 and K-4 had been extracted ten times with about 10 volumes of ether, then dried at about 50°C. and finally ground. The diet K-2 or K-3 was given to the day-old White Leghorn chicks for the first 4 days, then the diet K-1 was fed which is similar to the vitamin K-deficient diet E of Almquist and Stokstad ('36) and to the diet 417 of Dam and Glavind ('38, I). White Rock chicks received the diet K-1 as initial and sole basal diet because they were found to grow at a slightly greater rate than White Leghorn chicks. It should be noted that it is not necessary to start White Leghorns on diet K-2 or K-3; in our more

TABLE 1

*Composition of basal diets*

| ARTICLE OF DIET | RATION NO. K-1 | RATION NO. K-2 | RATION NO. K-3 | RATION NO. K-4 |
|---|---|---|---|---|
| | % | % | % | % |
| Cornstarch (dextrinized) | ... | ... | 71 | ... |
| Polished rice (ground) | 71 | 71 | ... | 71 |
| Crude casein (commercial) | ... | 17½ | 17½ | ... |
| Vitamin-free casein (Labco)[1] | ... | ... | ... | 17½ |
| Fish meal[2] (ether extracted) | 17½ | ... | ... | ... |
| Brewer's yeast (powdered)[3] | ... | 7½ | 7½ | ... |
| Brewer's yeast (ether extracted) | 7½ | ... | ... | 7½ |
| Salt mixture (Osborne and Mendel)[4] | 3 | 3 | 3 | 3 |
| Cod liver oil (Squibb) | 1 | 1 | 1 | 1 |

[1] Distributed by the Borden Co., New York, N. Y.

[2] Lighthouse brand, distributed by Wilbur-Ellis Co., San Francisco, Calif.

[3] Strain L distributed by Anheuser-Busch, Inc., St. Louis, Mo.

[4] J. Biol. Chem., vol. 15, p. 317 ('13); 0.04% of copper sulfate and 5% of manganous sulfate were added to the salt mixture.

recent experiments all the chicks were reared solely on diet K-1. The diet K-4 is identical with K-1 with the exception of the protein constituent which is Labco vitamin-free casein (Supplee et al., '36) in place of the fish meal of diet K-1; irrespective of the breed of the chicks, the ration K-4 was used as the initial and only basal diet.

The incidence of the vitamin K-deficiency symptoms, resulting from the feeding of the various basal rations, is recorded in table 2. The hemorrhagic syndrome, typical for the vitamin K-avitaminosis of the chick, was most frequently

found in the form of subcutaneous and intramuscular hemorrhages on the head, neck, breast, abdomen, back, wings and legs. The data presented show conclusively that diet K-1 contains little, if any, vitamin K. All the chicks on this diet died within 4 weeks unless they received a vitamin K supplement. It is also apparent that the diet K-3 contains a sufficient amount of vitamin K to prevent the occurrence of hemorrhages; therefore, it can be used only as an initial basal diet. The diet K-4, although identical with K-1 aside from the protein constituent, is evidently not suitable as a basal diet for vitamin K assays, in which a very pronounced deficiency is a prerequisite. Many chicks were found to be normal when this diet was used as the initial and sole basal ration for 23 days. Therefore, the Labco vitamin-free casein, the basal protein of this diet, either is not devoid of vitamin K or it interferes with the development of the hemorrhagic diathesis of the chick. Possibly prolonged extraction with ether of this brand of vitamin-free casein would result in a vitamin K-free product.

The data obtained from the feeding of the vitamin K-free ration K-1 are summarized in table 3. All the groups of experimental animals showing a numerical figure followed by $K_2$ received the ration K-2 as the initial diet and ration K-1 from the fifth day on; those marked '$K_3$' received first the diet K-3, and after 4 days the ration K-1; those marked '$K_1$' received the ration K-1 as the initial and sole diet. All the chicks were White Leghorns with the exception of those of lots 16, 17 and 18 which were White Rocks. The data of table 3 show that all the chicks became vitamin K-deficient within 15 days [2] and that only twenty-nine of the 1835 chicks

[2] Since this paper was submitted for publication, twenty-one additional lots of chicks were used in vitamin K assays, namely 484 White Rocks and 2002 White Leghorns; all these animals became vitamin K-deficient within 15 days, bringing the number of normally responding chicks to 4321. However, 100 White Leghorns (lot 39) obtained from a different hatchery did not develop the hemorrhagic diathesis in the 15-day period. In collaboration with Dr. Sibylle Tolksdorf of this Institute we found that these chicks had enlarged thyroid glands; whether they had other anatomical abnormalities is not known. Attempts are being made to confirm these observations and experiments are under way to demonstrate a relationship of endocrine glands to vitamin K metabolism.

TABLE 2

*Incidence of symptoms resulting from the feeding of the various basal rations*

| RATION | TOTAL NUMBER OF CHICKS | MORTALITY 1ST TO 10TH DAY | VISIBLE SYMPTOMS ON | | | | | | | | | | | | | 23RD DAY NORMAL BLOOD CLOTTING TIME AND NO SYMPTOMS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 11th day | 12th day | 13th day | 14th day | 15th day | 16th day | 17th day | 18th day | 19th day | 20th day | 21st day | 22nd day | 23rd day | |
| | | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % |
| K-1 | 267 [1] | 5.6 | 40.1 | 20.9 | 16.5 | 7.5 | 9.4 [5] | ... | ... | ... | ... | .. | .. | .. | .. | ... |
| K-2 or K-3 for 4 days, then K-1 | 1568 [2] | 6.7 | 16.2 | 26.7 | 25.1 | 19.5 | 5.8 [6] | ... | ... | ... | ... | .. | .. | .. | .. | ... |
| K-3 for 10 days, then K-1 | 100 [3] | 4.0 | ... | ... | 6.0 | 9.0 | 16.0 | 14.0 | 20.0 | 18.0 | 13.0 | .. | .. | .. | .. | ... |
| K-4 | 115 [4] | 2.6 | 2.6 | 10.5 | 17.4 | 1.7 | 8.7 | 19.1 | 1.7 | 3.5 | 3.5 | 5.2 | 0.9 | 2.6 | 4.3 | 15.7 |

[1] Composite of three lots.
[2] Composite of thirteen lots.
[3] Lot no. 11.
[4] Composite of five lots.
[5] Includes 1.9% with no visible symptoms, but with a coagulation time of more than 30 minutes.
[6] Includes 1.5% with no visible symptoms, but with a coagulation time of more than 30 minutes.

TABLE 3

*Incidence and type of symptoms resulting from the feeding of the vitamin K-free diet (K-1)*

| LOT NO. | TOTAL NUMBER OF CHICKS | MORTALITY 1st to 10th day | VISIBLE SYMPTOMS ON | | | | | NO SYMPTOMS BUT BCT [2] >30' | HEMORRHAGES ON | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 11th day | 12th day | 13th day | 14th day | 15th day | | Abdomen | Legs | Shoulders | Wings | Head | Breast | Side | Back | Neck |
| | | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % | % |
| 5-K$_1$ | 200 | 6.5 | 27.0 | 30.0 | 17.0 | 19.5 | ... | .. | 25.0 | 15.0 | 6.5 | 14.0 | 10.5 | 8.0 | .. | 7.0 | 7.5 |
| 6-K$_1$ | 100 | 8.0 | ... | 25.0 | 36.0 | 21.0 | 10.0 | .. | 28.0 | 14.0 | 12.0 | 14.0 | 6.0 | 6.0 | 3.0 | 8.0 | 1.0 |
| 7-K$_1$ | 103 | 3.9 | 22.4 | 30.1 | 25.2 | 18.4 | ... | .. | 35.8 | 17.5 | 6.8 | 9.7 | 6.8 | 7.8 | 1.0 | 9.7 | 1.0 |
| 8-K$_1$ | 103 | 9.7 | 28.2 | 35.9 | 17.5 | 8.7 | ... | .. | 33.0 | 17.5 | 5.8 | 8.8 | 8.8 | 7.8 | 2.9 | 3.8 | 1.9 |
| 9-K$_1$ | 101 | 3.0 | ... | 24.7 | 44.6 | 27.7 | ... | .. | 30.7 | 13.9 | 12.8 | 15.7 | 3.0 | 13.9 | 4.0 | 2.0 | 1.0 |
| 10-K$_1$ | 204 | 8.8 | 26.0 | 29.4 | 22.1 | 13.7 | ... | .. | 21.6 | 12.7 | 17.2 | 10.8 | 8.3 | 9.3 | 6.4 | 3.9 | 1.0 |
| 13[1]-K$_1$ | 101 | 2.0 | 5.0 | 23.7 | 34.6 | 29.7 | ... | 5.0 | 18.8 | 17.9 | 11.9 | 16.8 | 5.9 | 6.9 | 6.9 | 1.0 | 6.9 |
| 14-K$_1$ | 99 | 8.1 | 25.2 | 20.2 | 20.2 | 14.1 | 7.1 | 5.1 | 16.2 | 19.1 | 13.1 | 7.1 | 18.2 | 5.1 | 4.0 | 1.0 | 3.0 |
| 15-K$_1$ | 112 | 7.1 | 7.1 | 17.9 | 31.3 | 24.1 | 8.9 | 3.6 | 15.2 | 16.9 | 16.1 | 16.1 | 7.1 | 3.6 | 4.5 | 3.6 | 6.2 |
| 16-K$_1$ | 79 | 11.4 | 24.0 | 16.5 | 16.5 | 25.3 | 6.3 | .. | 19.0 | 12.6 | 17.7 | 22.8 | 5.1 | 1.3 | 3.8 | 3.8 | 2.5 |
| 17-K$_1$ | 80 | 7.5 | 68.8 | 11.3 | 6.2 | 6.2 | ... | .. | 20.0 | 15.0 | 25.0 | 16.3 | 3.7 | .. | 2.5 | 5.0 | 5.0 |
| 18-K$_1$ | 85 | 5.9 | 35.3 | 29.4 | 21.2 | 4.7 | 3.5 | .. | 34.1 | 11.8 | 17.6 | 10.6 | 8.3 | 1.2 | 3.5 | 3.5 | 3.5 |
| 19-K$_1$ | 166 | 7.2 | 9.6 | 35.6 | 18.7 | 21.1 | 6.0 | 1.8 | 17.5 | 18.1 | 14.5 | 13.3 | 10.8 | 4.8 | 8.4 | 2.4 | 1.2 |
| 20-K$_1$ | 100 | 3.0 | 3.0 | 20.0 | 37.0 | 28.0 | 5.0 | 4.0 | 46.0 | 12.0 | 13.0 | 6.0 | 1.0 | 3.0 | 5.0 | 5.0 | 2.0 |
| 21-K$_1$ | 100 | 7.0 | 19.0 | 24.0 | 18.0 | 8.0 | 21.0 | 3.0 | 13.0 | 14.0 | 20.0 | 10.0 | 20.0 | 6.0 | 4.0 | 1.0 | 2.0 |
| 22-K$_1$ | 102 | 4.0 | 21.5 | 21.5 | 20.6 | 10.8 | 16.7 | 4.9 | 11.8 | 18.6 | 9.8 | 7.8 | 31.3 | 3.0 | 5.8 | 1.0 | 2.0 |
| Chicks Total no. | 1835 | 120 | 361 | 474 | 437 | 326 | 88 | 29 | 436 | 283 | 245 | 227 | 180 | 109 | 77 | 73 | 56 |
| Chicks Per cent | 100 | 6.5 | 19.7 | 25.8 | 23.8 | 17.8 | 4.8 | 1.6 | 23.8 | 15.4 | 13.3 | 12.4 | 9.8 | 5.9 | 4.2 | 4.0 | 3.1 |

[1] Lot no. 11, see table 2; the chicks of lot no. 12 were not used for vitamin K studies.

[2] BCT, blood clotting time.

did not show clinical signs of vitamin K-avitaminosis other than a prolonged blood clotting time (BCT > 30′).

The blood clotting time was determined in a manner similar to the one described by Almquist and Stokstad ('37): a small wing vein was punctured with a fine sewing needle; the blood was allowed to flow into a micro-test tube (3.8 × 0.2 mm.; 0.1 mm. thickness). The tube was placed at once in a water thermostat with a temperature of 38 to 40°C. and shaken by a constant speed mechanism. As the clotting time we considered the length of time necessary for a solid clot to form so that the tube can be inverted without dislodgement of the clot. The same definition for the clotting time has been given by Hawkins and Whipple ('35).

The coagulation time of the normal chick is less than 6 minutes and that of the chick showing the typical vitamin K-deficiency symptoms and receiving solely ration K-1 is markedly prolonged. Usually, the tubes were discarded after 30 minutes; however, in many experiments wherein the tubes were left in the thermostat for 3 hours, no clots were formed. In one set of experiments in which the tubes were left in the thermostat over night, no clots were observed within 15 hours.

The coagulation time of the chicks on ration K-1 is always markedly prolonged before hemorrhages appear. We did not observe a single animal on diet K-1 with a normal blood clotting time and with the hemorrhagic syndrome. The data of table 3 show also that there is only a relatively slight variation in the incidence of the hemorrhages from lot to lot. However, the site of the hemorrhages varies markedly from group to group and appears to be accidental.

As soon as a chick on ration K-1 shows the hemorrhagic syndrome, it is used for the assay of a vitamin K preparation. Its symptoms and weight are recorded. The test substance is then given orally by pipette. The pipette used is a ¼ ml. hypodermic syringe graduated in 1/100 ml. to which a glass tube (4 × 0.3 mm.; 0.1 mm. thickness) has been attached by means of rubber tubing. The content of the pipette is intro-

duced into the chick's crop. The animal is then allowed to remain in a cage for 6 hours during which time it receives neither food nor water. Finally a blood sample is taken and the clotting time determined.

If the test substance contains vitamin K, the clotting power of the blood of the vitamin K-deficient chick will be restored within a very short time (Ansbacher, '38). All our tests are made in a 6-hour period, since we define the unit of vitamin K as the minimum amount necessary to render the blood clotting time of the vitamin K-deficient chick, weighing 70 to 100 gm., normal within 6 hours after administration. In the case of vitamin K concentrates, the product is fed in 0.10 ml. of cod liver oil.

Usually five chicks are employed for each concentration of the vitamin K preparation to be assayed, as shown in table 4 which records the biological activity of the vitamin K concentrate no. 14 prepared from alfalfa (Fernholz et al., '39). The data presented show that 1¼ mg. of this preparation is by definition equal to 1 unit of vitamin K. Parenthetically it may be added that the chicks fed the lowest dose of this concentrate did not die as a result of the vein puncture, although their clotting time was more than 30 minutes. They appeared to be much improved on the following day, but on the third day they were considerably weaker and death occurred within 5 days. The chicks to which the concentrate no. 14 had been given at the two highest levels showed a normal coagulation time for an entire week; after 12 days all these animals had a blood clotting time of more than 30 minutes and died from unchecked bleeding.

It should be noted also that the oil, used as a medium for administering vitamin K concentrates, appears to interfere to a small but noticeable degree with the potency of vitamin K. As shown in table 4, the biological activity of 1¼ mg. of the concentrate no. 14 fed in 0.05 ml. of cod liver oil was slightly greater than that of the same dose in 0.10 ml. This observation was made with many samples and the difference

TABLE 4

*Biological activity of vitamin K contrate no. 14*[1]

| QUANTITY | 62½ MG. IN 0.25 ML. CLO[2] | 50 MG. IN 0.20 ML. CLO | 6¼ MG. IN 0.25 ML. CLO | 5 MG. IN 0.20 ML. CLO | 3¾ MG. IN 0.15 ML. CLO | 2½ MG. IN 0.10 ML. CLO | 2 MG. IN 0.20 ML. CLO | 1¼ MG. IN 0.10 ML. CLO | 1¼ MG. IN 0.15 ML. CLO | 1¼ MG. IN 0.10 ML. CLO | 1¼ MG. IN 0.10 ML. CLO | 1¼ MG. IN 0.05 ML. CLO | 1 MG. IN 0.20 ML. CLO | 1 MG. IN 0.15 ML. CLO | ¾ MG. IN 0.15 ML. CLO | ½ MG. IN 0.10 ML. CLO |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Blood clotting time (minutes) of the experimental chicks after 6 hours | < ½ | < ½ | < ½ | < ½ | < ½ | < ½ | < ¼ | < 1 | < 3 | < 2 | < 2 | < 1 | < 4 | < 2 | > 30 | < 25 |
| | < ½ | < ½ | < 1 | < ½ | < 1 | < 1 | < 3 | < 3 | < 4 | < 5 | < 6 | < 1 | < 8 | < 5 | > 30 | > 30 |
| | < ½ | < ½ | < 1 | < ½ | < 1 | < 2 | < 4 | < 5 | < 5 | < 5 | < 6 | < 2 | > 30 | < 5 | > 30 | > 30 |
| | < ½ | < ½ | < 2 | < 1 | < 2 | < 2 | < 4 | < 6 | < 5 | < 5 | < 8 | < 3 | > 30 | < 15 | > 30 | > 30 |
| | < 1 | < 2 | < 2 | < 1 | < 2 | < 3 | < 6 | < 6 | < 6 | < 6 | < 15 | < 3 | > 30 | > 30 | > 30 | > 30 |

[1] The biological activity of the freshly prepared concentrate is shown. The vitamin K potency of concentrates gradually diminishes, and this particular sample was found to have lost about 50% of its activity during storage at a temperature of approximately 5°C. for 2 months.

[2] CLO, cod liver oil.

in potency observed was especially pronounced when 0.20 ml. of the oil was used as a vehicle in place of 0.10 ml.

In the earlier experiments we determined the blood clotting time of every chick showing the hemorrhagic syndrome. Usually, these determinations were made within 2 hours after the administration of a vitamin K preparation. Because we did not observe a single experimental animal with a normal coagulation time at this stage of the test, we omitted these determinations in the more recent assays. If a chick's coagulation time is to be determined twice during the experimental period, it is advisable to tap the left wing for the first blood clotting time determination and the right wing for the second one, since we observed a greater mortality from the puncture of a right wing vein. Vitamin K-deficient chicks usually die as a result of a vein puncture, unless they receive a potent vitamin K concentrate either shortly before or immediately after being bled.

To date more than 100 vitamin K samples have been tested by our biological assay method. The experience obtained in these determinations permits us to use a small number of experimental animals. The data of the individual chicks having received a preparation at a certain level, will give a clue as to where the concentration corresponding to about 1 unit of vitamin K lies. It was observed, namely, that the clotting time of most of the test animals will be reduced during the 6-hour test period to less than $\frac{1}{2}$ minute, when several units of vitamin K are administered. Six hours after the feeding of 1 unit of vitamin K, the chicks are normal with respect to clotting, but one or two animals may be found with a slightly prolonged coagulation time of 6 to 15 minutes. If less than 1 but more than $\frac{1}{2}$ unit of vitamin K is fed, several chicks will retain their prolonged clotting time. If $\frac{1}{2}$ to $\frac{1}{4}$ unit is given, the clotting time remains prolonged during the 6-hour test period, but the animals will survive the vein puncture made in the determination of the coagulation power of the blood. If less than $\frac{1}{4}$ unit is administered, the clotting

time will not be changed and the animals will die from unchecked bleeding.

In view of the numerous data published by the Danish investigators Schønheyder, Dam and co-workers, it seemed desirable to obtain comparable data of our unit, defined above, and that of Dam, who gave the following definition (Dam, '37): a unit of vitamin K is that amount which is to be administered to the test animal per gram of body weight in order to reduce its value $R \geqq 200$ to 1 ($R = \frac{}{Kn}$, K equals concentration of the tissue extract (Dam and Glavind, '38, II) necessary to cause clotting of the blood plasma in 3 minutes;

TABLE 5

*Biological data of vitamin K standard (500 Dam units per gram)*

| AMOUNT | BLOOD CLOTTING TIME OF INDIVIDUAL CHICKS 6 HOURS AFTER ORAL ADMINISTRATION | | | | | |
|---|---|---|---|---|---|---|
| *mg.* | *minutes* | | | | | |
| 270[1] | < ½ | < ½ | < ½ | < ½ | < ½ | < ½ |
| 100 | < ½ | < ½ | < ½ | < ½ | < ½ | < ½ |
| 40 | < 2 | < 2 | < 4 | < 6 | < 7 | < 8 |
| 38 | < 1 | < 1 | < 2 | < 6 | < 10 | < 25 |
| 35 | < 1 | < 2 | < 8 | < 20 | > 30 | > 30 |
| 34 | < 3 | < 4 | > 30 | > 30 | > 30 | > 30 |
| 30 | < 5 | < 6 | > 30 | > 30 | > 30 | > 30 |
| 25 | < 8 | > 30 | > 30 | > 30 | > 30 | > 30 |
| 10 | > 30 | > 30 | > 30 | > 30 | > 30 | > 30 |

[1] One tablet.

Kn equals the corresponding concentration for normal plasma). In order to enable us to make a comparison of the Dam unit of vitamin K with ours, Dr. H. Dam of the Biochemical Institute of the University of Copenhagen, Denmark, sent[3] to us samples of his vitamin K standard, "a selected dried spinach powder formed into tablets and stored under precautions securing stability." This standard preparation, found to contain 500 units of vitamin K per gram by Dam and Glavind ('38, I), was biologically assayed and the results are recorded in table 5. For the biological assay, the tablets were

[3] We wish to take this opportunity of thanking Doctor Dam for his kindness in sending us his vitamin K standard preparation.

cut with a knife into small pieces, and weighed amounts were introduced into the gullets of vitamin K-deficient chicks by means of small forceps and pushed into the crops. Finally the chicks were given 1 ml. of warm water and their blood clotting time was determined in the usual manner after 6 hours. It is apparent from the data of table 5 that 100 mg. of the standard contain several units, since the clotting time of the chicks was reduced to less than ½ minute within the 6-hour test period. The data as a whole show clearly that 40 mg. of the standard preparation have a potency of 1 unit of vitamin K. Therefore, the vitamin K standard contains 25 units per gram, and since the potency of 1 gm. was found to be 500 Dam units by the European workers, our unit is equal to 20 Dam units of vitamin K.

Clinical investigations are now being made with highly potent vitamin K concentrates prepared in this Institute. The dosage employed in these experiments is based upon the unit of vitamin K, as defined in this report.

## SUMMARY

A comparative study of various basal diets for vitamin K assays was made on 2050 chicks.

A ration was found which permitted the development of severe vitamin K-deficiency in all the animals within 15 days.

A 6-hour curative blood clotting method for the determination of vitamin K was developed.

The unit of vitamin K was defined and the biological response resulting from the administration of unit amounts of vitamin K reported.

The unit, as defined, was found to equal 20 Dam units of vitamin K.

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# AN EXPERIMENTAL DETERMINATION OF THE MINIMUM VITAMIN A REQUIREMENTS OF NORMAL ADULTS [1]

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TWO FIGURES

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Present knowledge of the vitamin A requirements of man has been summarized very recently in a review article by Booher ('38). Articles by Bessey and Wolbach ('38) and by Clausen ('38) summarizing the physiological functions of vitamin A and the pathological signs of vitamin A deficiency have also been published recently. The timeliness of these articles dismisses the need for extended review of the literature here.

The development of nutritional night-blindness is generally recognized as the earliest tangible sign of an inadequate intake of vitamin A. For a discussion of the chemical basis of vision, including the role of vitamin A in the visual processes, the reader is referred to articles by Hecht ('37), by Wald ('35), by Wald and Clark ('37) and by Krause and Sidwell ('38). The daily intake of vitamin A or carotene which will just prevent the appearance of night-blindness is a physiologic minimum requirement and does not provide for the accumulation of vitamin A reserves in the body. According to Guilbert, Miller and Hughes ('37), the physiologic minimum requirement of vitamin A for animals (cattle, sheep and

[1] This research was supported by an appropriation from Bankhead-Jones funds (Bankhead-Jones Act of June 29, 1935).

swine) may be supplied by a daily intake of 25 to 30 micrograms (approximately 40 to 50 international units) of carotene, or by about 6 to 8 micrograms (about 18 to 24 units)[2] of vitamin A per kilogram of body weight. These levels of carotene or vitamin A intake sufficed for excellent gains in weight and for the maintenance of a thrifty physical appearance of the animals for indefinite periods, but did not provide any margin for storage of vitamin A in the liver nor for the needs of normal reproduction and lactation.

Edmund and Clemmesen ('37) have reported a study on the vitamin A requirements of young adult men as carried out at the state prison in Nyborg (Denmark). They found that the fixed prison allowance which furnished an average of 1225 international units per day (about 17 to 19 units per kilogram of body weight) did not prevent seasonal oscillation in the power of visual distinction. Another group of young men who received the regular prison fare plus ½ liter of whole fluid milk daily for a 6-month (autumn and winter) experimental period showed normal powers of visual distinction at all seasons. It was estimated that this latter group received an average total of about 1400 international units of vitamin A (about 20 to 22 units per kilogram of body weight) per day. In view of the fact that the vitamin A intake was estimated for food consumed by groups of persons, it was not possible to determine precisely the individual vitamin A intake nor the variations in requirements.

## EXPERIMENTAL

Five well-nourished adult subjects (two male and three female) employed at the Bureau of Home Economics served as experimental subjects for the determination of the minimum requirements of vitamin A and carotene.

*The basal diet.* Each subject received throughout the duration of the experiments (5 to 6 months) a basal weighed diet

[2] This conversion was made by the authors on the assumption that 1 gm. of pure vitamin A is equivalent to about 3,000,000 international units as indicated in the report of Holmes and Corbet (J. Am. Chem. Soc., vol. 59, p. 2042, 1937).

of ordinary food items adequate in all known food essentials except vitamin A, which was included in as small amounts as was practicable. Both the kinds and amounts of food were varied from day to day in order that any possible unusual effect of particular food combinations on the utilization of vitamin A or carotene might be minimized and in order that the subjects might be as contented with the diet as possible.

All the food was prepared and weighed in a special kitchen at the Bureau of Home Economics. The daily diets of the subjects were supplied in quantities which would satisfy their respective appetites and would provide adequate and practically constant calories, protein, fat, calcium, phosphorus, iron and the known vitamins. At the same time, the daily intake of vitamin A was kept at as low a level as possible. In order to maintain an adequate level of calcium and phosphorus, milk was of necessity included in the diet. Separated milk containing less than 0.02% butterfat which was used in this experiment is reported to contain vitamin A in traces only. However, it was found by biological assay with rats that appreciable amounts were present in such milk (about 80 units per quart). Certain other foods containing small quantities of vitamin A were included to give color and flavor to an otherwise pale and bland diet. Vitamin A-free bread [3] made of white flour, hydrogenated cottonseed oil, sugar and yeast was especially baked for use in these experiments.

The menus for the basal diet were chosen from the following list of foods:

Meats: Bacon, chicken, fish (lean), ham, lamb, pork and veal (all visible fat removed except in the case of bacon).

Vegetables: Asparagus (bleached), beans (dried), beets, cabbage (bleached), cauliflower, celery (bleached hearts), corn (white), cucumbers, mushrooms, onions, parsnips, potatoes (white) and turnips (white).

Fruits: Apples, cranberries, grapefruit, grape juice, grapes (white), lemons, pears and strawberries.

[3] Prepared by Mrs. Adelaide Freeman, Bureau of Home Economics, U. S. Department of Agriculture.

Miscellaneous: Cocoa, coconut, hydrogenated cottonseed oil, egg white, flour (white), gelatin, honey, macaroni, milk (fat content less than 0.02%), margarine (made with vegetable oil and containing no added vitamin A), white rice, saltines, sugar and tapioca.

Dried baker's yeast was used to supplement the dietary supply of thiamin and riboflavin; small amounts of ferric ammonium citrate or ferrous carbonate were added to insure an abundance of iron; iodine was supplied in the iodized salt used for seasoning; either viosterol or irradiation with the mercury vapor lamp supplied vitamin D. Vitamin C was provided in liberal amounts in the fruits and vegetables.

Breakfast was the least variable of the meals consisting of grapefruit, toast, bacon, margarine, honey, skim milk and black coffee. A representative dinner would be: chicken, potatoes and margarine, cauliflower, a small portion of cranberry sauce, pears and skim milk. Supper: navy bean soup, saltines, a small serving of apple, celery, and nut salad with lemon juice dressing, cocoa and angel food cake.

The average composition of the daily food intakes of each subject is shown in table 1. Average figures for the composition of the food items were used in making the dietary calculations.

*Measurements of dark adaptation.* Before and during the beginning of the period of low vitamin A intake, from ten to fifteen tests of dark adaptation were made on each subject in order to establish curves of normal response. These tests were made with the visual adaptometer designed and described by Hecht and Shlaer ('38). Measurements made with this instrument have already appeared in an article by Haig, Hecht and Patek ('38) in connection with a study of the utilization of vitamin A concentrates by an individual suffering from cirrhosis of the liver.

The dark adaptation measurements were preceded by a 3-minute period of light adaptation. The brightness of the light-adapting screen located 50 cm. directly in front of the subject was 1400 millilamberts. The brightness of this screen

and the length of the light adaptation period were constant for all tests.

The light used in the determination of threshold values emanated from a filament lamp, the brightness of which was calibrated at frequent intervals. Gross gradations of the light intensity were obtained by the use of suitable calibrated neutral filters. The finer gradations were made by means of a carefully calibrated, movable, photometric wedge. In order to make the difference between the rod curve and cone curve

TABLE 1

*Average composition of basal weighed diets adequate in all other respects except vitamin A (quantities refer to daily intakes)*

| SUBJECT | TOTAL CALORIES | PROTEIN | FAT | MINERALS [1] | | | VITAMINS [2] | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Calcium | Phosphorus | Iron | A | Thiamin | Ascorbic acid | Riboflavin |
| | | *gm.* | *gm.* | *gm.* | *gm.* | *gm.* | *I.U.* | *I.U.* | *I.U.* | *S.U.* |
| I | 2380 | 66 | 74 | 1.13 | 1.37 | 0.026 | 93 | 600 | 2800 | 870 |
| II | 2420 | 89 | 90 | 1.13 | 1.44 | 0.026 | 103 | 590 | 3200 | 960 |
| III | 2260 | 72 | 76 | 1.22 | 1.39 | 0.025 | 91 | 610 | 2900 | 960 |
| IV | 2260 | 82 | 82 | 1.22 | 1.46 | 0.024 | 88 | 560 | 2800 | 900 |
| V | 2160 | 78 | 76 | 1.15 | 1.39 | 0.023 | 90 | 550 | 2300 | 890 |

[1] Ferric ammonium citrate and ferrous carbonate supplements mentioned in text are included in these averages.

[2] Values for vitamin A, thiamin and ascorbic acid are given in terms of international units, riboflavin values in terms of Sherman units. The thiamin and riboflavin supplements from the yeast are included in these averages. Vitamin D was supplied either by administration of viosterol or by irradiation of the skin with emanations from a mercury quartz lamp.

more pronounced, the light used for the test spot was restricted to the violet region of the spectrum by using a filter transmitting only wavelengths below 460 mμ. The duration of the light stimulus was limited to 0.2 second by means of a photographic shutter.

For subjects I, II and III, dark adaptation was measured at a position 7° from the fovea on the nasal side of the retina; for subject IV, the retinal image was central, and for subject V, the retinal image was placed at 15° temporal. In all cases, the test spot was a circle whose diameter subtended a 3° visual angle. On three subjects the tests were made regularly on

the right eye, and on the others on the left eye. However, measurements made on both eyes during the period of night-blindness demonstrated that both retinas were equally affected by lack of vitamin A.

Three curves of the dark adaptation response for subject I showing the normal curve, the curve of response 36 days after the beginning of the period of low vitamin A intake a short time after definite signs of night-blindness appeared, and a

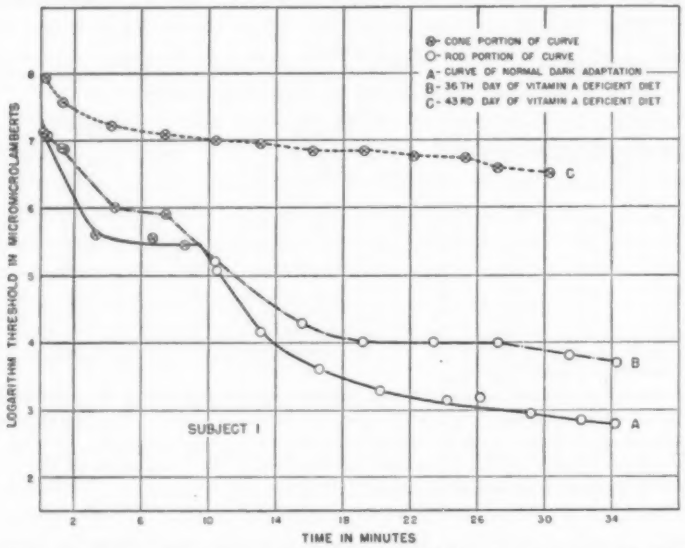
CONE PORTION OF CURVE
ROD PORTION OF CURVE
A- CURVE OF NORMAL DARK ADAPTATION
B- 36 TH DAY OF VITAMIN A DEFICIENT DIET
C- 43 RD DAY OF VITAMIN A DEFICIENT DIET
LOGARITHM THRESHOLD IN MICROMICROLAMBERTS
SUBJECT I
TIME IN MINUTES
A
B
C

Fig. 1 Dark adaptation curves for subject I showing progress of night-blindness produced by the vitamin A-low diet.

curve determined 43 days after the beginning of the period of low vitamin A intake when a very marked degree of night-blindness had developed are shown in figure 1. It is apparent from this set of curves that both the cones and the rods are affected as the result of an inadequate intake of vitamin A. When the degree of night-blindness was most marked (curve C) the subject was subjectively aware of a restricted visual field. This observation was confirmed by actual measure-

ments [4] of the visual field with a perimeter. In comparison with this subject's normal visual field, the visual field area coexistent with the dark adaptation response represented by curve B (fig. 1) was perceptibly restricted. The magnitude of the restriction at this stage of vitamin A-deficiency, however, was not such as, of itself, would have definitely indicated an abnormal visual field area.

Ophthalmological examinations [5] made with a slit-lamp revealed no evidence of keratinization of the epithelial structures of the eye at any stage of night-blindness encountered in the course of these experiments.

TABLE 2

*General description of experimental subjects*

| SUBJECT | SEX | AGE IN YEARS | HEIGHT IN CENTIMETERS | AVERAGE WEIGHT IN KILOGRAMS | AVERAGE NORMAL LIGHT THRESHOLDS [1] AFTER | |
|---|---|---|---|---|---|---|
| | | | | | 6-minute dark adaptation | 30-minute dark adaptation |
| | | | | | *log micromicro-lamberts* | *log micromicro-lamberts* |
| I | M | 25 | 168.9 | 53.1±0.8 | 5.60±0.15 | 2.96±0.12 |
| II | M | 21 | 183.5 | 70.0±0.7 | 5.53±0.08 | 2.78±0.14 |
| III | F | 39 | 165.7 | 60.0±0.4 | 5.67±0.08 | 3.06±0.05 |
| IV | F | 30 | 163.8 | 51.6±0.6 | 5.87±0.15 | 3.87±0.20 |
| V | F | 40 | 164.5 | 59.7±0.4 | 5.85±0.13 | 2.83±0.10 |

[1] Subjects I, II and III were tested at a position 7° nasal; subject IV, central; subject V, 15° temporal.

*Description of experimental subjects.* A general description including sex, age, height, average body weight for the total experimental period and the normal light thresholds corresponding to 6- and to 30-minute periods of dark adaptation following a 3-minute period of light adaptation is given for each of the experimental subjects in table 2. The body weights shown in this table are averages of approximately weekly weighings, taken just before the noon meal. The male

[4] The visual field measurements were made by Dr. Louise Sloan, Wilmer Eye Clinic, Baltimore.

[5] These examinations were made by Drs. Alan Woods and William Rowland, Wilmer Eye Clinic, Baltimore.

subjects were weighed without coats. The height and weight of all subjects were taken without shoes.

*Vitamin A value of cod liver oil and carotene supplements.* The cod liver oil supplements used in these experiments were all taken from a single sample. The vitamin A value of this oil was 3000 international units per gram as determined by biological assay with rats using crystalline beta-carotene [6] (in benzene the $E^{1\%}_{1\,cm.}$ values [7] at 430, 462 and 491 mμ were 1340, 2200 and 1930, respectively, as calculated from the equation $E^{1\%}_{1\,cm.} = \frac{1}{c \times d} \log_{10} \frac{I_0}{I}$ ) as the standard of reference.

The vitamin A value of the commercial carotene used to supplement the diets was determined by careful biological assay [8] with rats using both pure beta-carotene and U.S.P. reference cod liver oil as standards of reference. According to this assay 0.6 microgram of the commercial carotene used induced growth in vitamin A assay rats not significantly different from that induced by 0.6 microgram of crystalline beta-carotene and/or 1 unit of U.S.P. reference cod liver oil. Chromatographic analysis [9] of the commercial carotene indicated that it contained 93.4% of carotene, of which 85% was beta-carotene and 15% was alpha-carotene. The remaining 6.6% of the material consisted of an unidentified pigment.

In order to effect complete solution of the commercial carotene in cottonseed oil so that precise aliquots could be weighed out for vitamin A assay rats and for the experimental subjects, the requisite amount of carotene was first dissolved in purified light petroleum ether (ligroin, boiling at 30° to 60°C.) and the cottonseed oil added subsequently. This solution (containing 60 to 80 mg. per 100 gm. of cottonseed oil) was

[6] The crystalline beta-carotene was purchased from the S. M. A. Corporation, Cleveland, Ohio.

[7] The spectrographic analyses of the beta-carotene were made by Mr. H. G. Wiseman, Bureau of Dairy Industry, U. S. Department of Agriculture.

[8] We are indebted to Rosemary Loughlin, Bureau of Home Economics, U. S. Department of Agriculture, for valuable assistance in making the biological assays of the carotene and cod liver oil supplements.

[9] The chromatographic analysis of the commercial carotene was made by Mr. L. A. Shinn, Bureau of Dairy Industry, U. S. Department of Agriculture.

then subjected to gradually diminishing pressures (temperature 25°C.) until the petroleum ether was all removed, as indicated by a constant weight of the solution. All carotene solutions were freshly prepared each week and stored at a temperature of 0°C. No loss of vitamin A activity in the concentrated carotene solutions stored under these experimental conditions could be detected by careful rat-growth assays.

*Determination of vitamin A requirements.* Subjects I to V showed unmistakable signs of impaired dark adaptation after 29, 27, 16, 124 and 39 days, respectively, on the vitamin A low diet which varied in vitamin A content only between the limits of about 90 and 100 international units per day. It is quite certain from these observations that ostensibly normal persons vary widely in bodily reserves of vitamin A. An increment of 0.5 log unit in the respective averages of the normal light threshold measurements, taken after 30 minutes dark adaptation, was taken as unmistakable evidence of induced hemeralopia. None of the subjects who showed this degree of hemeralopia ever showed threshold values within their respective normal limits unless vitamin A or carotene was administered.

When the subjects' average light threshold values following 30 minutes dark adaptation had attained values on the order of 1.0 log unit or more in excess of their respective normal threshold values, the administration of vitamin A active materials was begun. The time required for the development of this more marked degree of hemeralopia varied for the five subjects studied between the limits of 5 and 16 weeks on the vitamin A deficient diet. Each subject was continued on the vitamin A low diet supplemented either by vitamin A in the form of cod liver oil or by commercial carotene in cottonseed oil administered each day before lunch.

The first dose of cod liver oil, which was less than adequate to restore normal dark adaptation, nevertheless induced definite improvement in dark adaptation within 2 to 3 hours. This improvement was only temporary, however, and repre-

sented only a partial cure, and by the end of 24 hours the light threshold had risen appreciably, though not to the level preceding the cod liver oil administration. A given daily intake of cod liver oil less than was required for complete restoration of normal dark adaptation improved the light sense with each dose for a period of several days but finally reached a practically stationary state. Prolongation of administration of a given inadequate dose of vitamin A for a period of 2 to 3 weeks did not result in any further improvement in dark adaptation beyond that attained during the first several days. The daily vitamin A intake was not considered adequate until the subjects were able to maintain a light threshold within their respective normal limits at all times for a period of a week. It would seem, therefore, that the blood of vitamin A-deficient subjects maintained a level of vitamin A commensurate with the vitamin A supply in the food, or perhaps with the vitamin A content of the tissues maintained by that in the food.

The daily intake of vitamin A (in the form of cod liver oil plus the small vitamin A value supplied by the food) necessary for the maintenance of normal dark adaptation varied for the five subjects studied between the limits of 25 and 55 international units per kilogram of body weight.

*Determination of carotene requirements.* Subsequent to the determination of the minimum vitamin A requirements of four of the five experimental subjects in terms of international units supplied by cod liver oil, a similar procedure was followed in determining the minimum vitamin A requirements of these subjects in terms of crystalline carotene. For subject IV the vitamin A requirement was determined subsequent to the determination of the carotene requirement.

After establishment of definitely impaired dark adaptation by withholding or withdrawing all vitamin A intake except that carried by the basal vitamin A deficient diet, graded quantities of the crystalline carotene dissolved in cottonseed oil were administered to each subject. The minimum daily intake of carotene necessary for the support of normal dark

TABLE 3

*Summary of data on the minimum vitamin A and carotene requirements per day for maintenance of normal dark adaptation*

| SUBJECT | IMPAIRED DARK ADAPTATION APPEARED AFTER | LIGHT THRESHOLD VALUES WHEN VITAMIN A SUPPLEMENTS WERE STARTED [1] | | AVERAGE VITAMIN A VALUE OF BASAL DIET | DAILY REQUIREMENT FOR NORMAL DARK ADAPTATION | | | | | | RATIO: CAROTENE REQUIREMENT / VITAMIN A REQUIREMENT |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Vitamin A | | | Carotene in cottonseed oil | | | |
| | | 6-minute dark adaptation | 30-minute dark adaptation | | Vitamin A value of cod liver oil suppl. | Total vitamin A intake | Vitamin A intake per kilogram | Vitamin A value of carotene suppl. | Total vitamin A intake | Vitamin A intake per kilogram | |
| | *days* | *log micromicro-lamberts* | *log micromicro-lamberts* | *I.U.* | *I.U.* | *I.U.* | *I.U.* | *I.U.* | *I.U.* | *I.U.* | |
| I | 29 | 7.05 | no rod curves [2] | 93 | 1800 | 1893 | 35.7 | 2950 | 3043 | 57.4 | 1.61 |
| II | 27 | 6.16 | 3.80 | 103 | 3800 | 3903 | 55.8 | 7080 | 7183 | 102.6 | 1.84 |
| III | 16 | 6.12 | 3.87 | 91 | 3050 | 3141 | 52.4 | 5310 | 5401 | 90.0 | 1.72 |
| IV | 124 | 6.10 | 4.74 | 88 | 1200 | 1288 | 24.8 | 2124 | 2212 | 42.5 | 1.71 |
| V | 39 | 6.00 | 3.55 | 90 | 1970 | 2060 | 34.3 | 3540 | 3630 | 60.5 | 1.76 |

[1] Subjects I, II and III were tested at a position 7° nasal; subject IV, central; subject V, 15° temporal.

[2] As shown in figure 1 (curve C) the rod structures were not functioning during the period of very marked impairment of dark adaptation.

adaptation was determined for each subject in accordance with the procedure described for the determination of physiologic minimum requirements for vitamin A in the form of cod liver oil.

The daily intake of carotene (expressed in terms of international units of vitamin A) necessary for the maintenance of normal dark adaptation varied for the five subjects studied

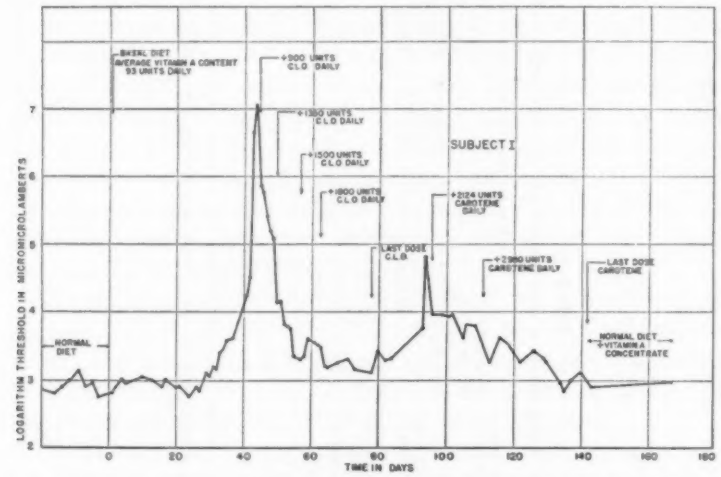


**Fig. 2** Graphic representation of the experimental procedure followed in determining the minimum vitamin A and carotene requirements. Data shown refer to subject I. Ordinates refer to threshold values after 30 minutes of dark adaptation. Vitamin units refer in all cases to international unit values.

between the limits of 43 and 103 units per kilogram of body weight.

The experimental procedure involved in determining the minimum vitamin A and carotene requirements of the five subjects is depicted in chronological detail for one subject (subject I) in figure 2. The minimum vitamin A and carotene requirements, respectively, for each subject are summarized in table 3.

## DISCUSSION

It is quite evident that the physiologic minimum requirements for vitamin A and/or carotene vary considerably among ostensibly normal adults. However, the ratios of the units of carotene to the units of vitamin A, respectively, which were required for normal dark adaptation in these subjects were almost constant (table 3). Unit for unit, the carotene in cottonseed oil was only about 50 to 60% as efficient in maintaining normal dark adaptation as vitamin A in the form of cod liver oil. In other words, the vitamin A potency of carotene in cottonseed oil, as determined by rat-growth assays, is only about 60% efficient in supporting dark adaptation in humans. Whether this represents a species difference in the absorption of carotene, or a species difference in the conversion of carotene into vitamin A, or a difference in the utilization of carotene for growth and dark adaptation was not indicated by the present experiments.

The human adult would appear to require somewhat higher absolute levels of either vitamin A or carotene per unit of body weight than cattle, sheep or swine. However, the estimates of the minimum vitamin A requirements of the domestic farm animals as determined by Guilbert et al. ('37) were based on needs for the prevention of gross signs of night-blindness, while those of human adults are based on needs for the prevention of a lesser degree of impaired dark adaptation. This explanation of the higher requirement levels for the human subjects reported here as compared with those reported for cattle, sheep and swine has ample experimental support. The levels of either vitamin A or carotene required to restore grossly impaired dark adaptation to a state which would appear normal with a less sensitive criterion (the biophotometer, for example) was on the order of 20% less than was required for restoration of a completely normal cone-rod dark adaptation response as measured with the visual adaptometer.

Measurements of dark adaptation were made with the biophotometer according to the method outlined by Jeans, Blanch-

ard and Zentmire ('37) on the experimental subjects at various stages of vitamin A deficiency. Only marked degrees of night-blindness could be detected with this instrument, an explanation for which may be seen by reference to figure 1. Curves B and C in this figure clearly depict a moderate and a marked degree of impaired dark adaptation, respectively, as measured with the visual adaptometer. The 'critical measurements,' as made with the biophotometer, correspond to the readings shown in the three curves taken immediately (within a 20-second interval) after the light adaptation period. It is evident that the light threshold value measured within this time interval is not essentially different in curve B from that in the normal curve (curve A).

## SUMMARY

Five adult subjects maintained on weighed diets adequate in all known food essentials except vitamin A (the average daily intake of which was not greater than 103 international units) showed unmistakable signs of impaired dark adaptation after 16, 27, 29, 39 and 124 days, respectively.

Impaired dark adaptation as measured with the visual adaptometer was the earliest definite ocular abnormality observed as a result of vitamin A deficiency.

The daily intake of vitamin A (in the form of cod liver oil plus the small vitamin A value supplied by the food) necessary for the maintenance of normal dark adaptation varied for the five subjects studied between the limits of 25 and 55 international units per kilogram of body weight.

The daily intake of carotene (expressed in terms of international units of vitamin A) necessary for the maintenance of normal dark adaptation varied for the five subjects studied between the limits of 43 and 103 units per kilogram of body weight.

The ratios of the requirements in terms of international units of carotene to the requirements in terms of international units of vitamin A in the form of cod liver oil were remarkably constant for all subjects. Unit for unit the carotene in

cottonseed oil was about 50 to 60% as effective in supporting normal dark adaptation as vitamin A in the form of cod liver oil.

ACKNOWLEDGMENTS

We are especially indebted to Dr. Selig Hecht and his associates at Columbia University for the timely introduction of the visual adaptometer and for their continued interest in this research, and to our many associates at the Bureau of Home Economics who have served as experimental subjects or who have contributed to the culmination of this work in a variety of ways.

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# FLUOROSIS IN RATS DUE TO CONTAMINATION WITH FLUORINE OF COMMERCIAL CASEIN. THE EFFECTS OF DARKNESS AND OF CONTROLLED RADIATION UPON THE PATHOLOGY OF THE TEETH[1]

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FIVE FIGURES

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## INTRODUCTION

During the past 18 months, October, 1936 to April, 1938, studies have been made of the effects of isolated radiation in the visible and infra red spectrum upon growth and reproduction in three succeeding generations of rats. Three rooms were designed in which it was possible to give to rats continuous exposure to radiation of approximately equal energy in the visible, the near infra red and the far infra red regions of the spectrum. A fourth room was kept completely dark. All rooms were kept at constant temperature. A full description of these rooms will be given in a later paper. It is the purpose of this paper to describe certain abnormalities of the teeth which were observed in one group of rats.

Two standard diets were used in these experiments: the Sherman B diet (Sherman and Campbell, '24) and also the Standard Diet no. 1 of McCollum (Evans, '22).

[1] Aided by a grant from the Committee on the Effects of Radiation upon Living Organisms, Divisions of Biology and Agriculture, National Research Council, and by a grant from the Carnegie Corporation of New York.

No attention was paid, until the experiments had been in progress for 1 year, to the teeth of the rats. They then obtruded themselves on our notice by the observation that certain rats were caught, by their upper incisors, upon the wires of the cage and were unable to extricate themselves. Examination showed that serious abnormalities of the teeth were developing in all the rats fed on the 'McCollum' diet (see footnote p. 336), while the teeth of a large group fed on the Sherman B diet were completely normal. It became evident that the abnormalities observed fitted the picture of fluorine poisoning described by McCollum ('25), Smith and Lantz ('33) and other writers though it was not at all obvious to us at first how the rats, which were fed on what we considered a standard normal diet, could have developed fluorosis.

## EXAMINATION OF DIET INGREDIENTS AND OF THE TEETH OF THE RATS FOR FLUORINE

*a. Diet ingredients.* In comparing the two diets,

| *Sherman B* | | *McCollum no. 1* | *per cent* |
|---|---|---|---|
| Whole wheat flour | 2/3 | Whole wheat flour | 67.5 |
| Klim | 1/3 | Casein | 15.0 |
| NaCl | 2% of the whole wheat flour | Klim | 10.0 |
| | | NaCl | 1.0 |
| | | $CaCO_3$ | 1.5 |
| | | Butterfat | 5.0 |

it is evident that only three ingredients are present in the 'McCollum' diet which are not used in the Sherman B mixture —namely casein, $CaCO_3$ and butterfat.

Spectrographic analyses of these three materials were carried out by Dr. L. T. Steadman of the division of radiology, department of medicine, School of Medicine and Dentistry.

The technic of Churchill ('31) was adopted. By comparing the spectra of a series of standards with the casein spectrum (fig. 1), it was shown that the casein contained approximately 0.1% fluorine. This figure was adjudged to represent the true value within a factor of 2.

Because the contamination of fluorine in the casein was so marked, it seemed advisable to obtain chemical analyses as

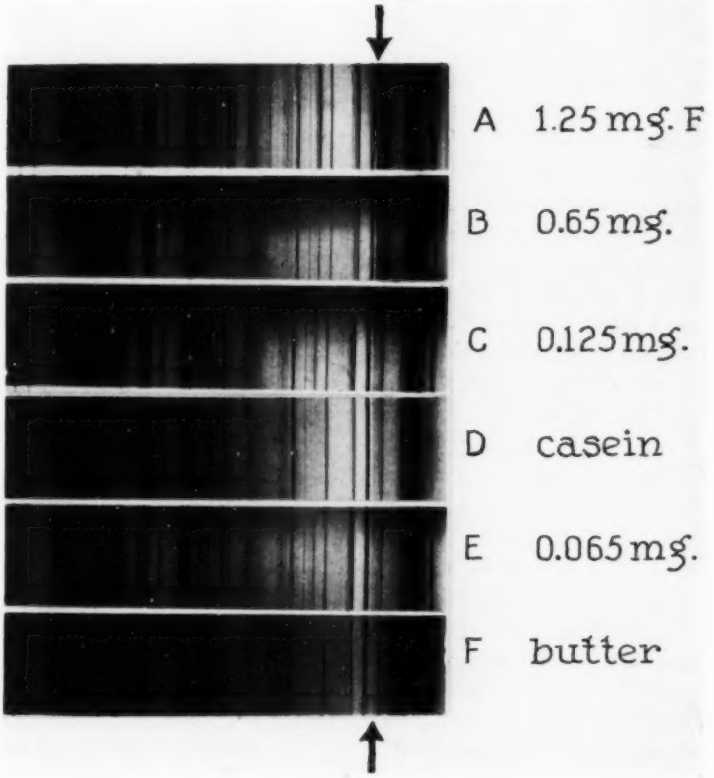


Fig. 1 Spectra showing $CaF_2$ bands at 5291 Å (between the arrow points). The spectra of a series of standards composed of fluorine-free whole powdered milk and varying amounts of NaF (A, B, C and E) may be compared with casein (D). The band is very dark in A, very light in E and absent in F (butter).

a check on the spectrographic data. Dr. G. H. Ellis of the Cornell University Agriculture Experiment Station kindly furnished these analyses using the methods of Willard and Winter ('33) and of Sanchis ('34). The results are as follows:

| *G. casein sample* | *G. CaO added* | *Per cent fluorine* |
|---|---|---|
| 10 | 1 | 0.20 |
| 5 | 1 | 0.19 |
| 5 | 0.5 | 0.19 |

Since the diet contained 15% of casein, the approximate fluorine content of the diet must have been 0.03 %. We have no means of knowing whether each batch of casein used during the year had been similarly contaminated, but if it had been, a rat ingesting 10 gm. of this diet [2] per day would obtain a daily dosage of 3 mg. of fluorine.

*b. Teeth of rats.* The incisors were taken from the jaws of ten rats, five with marked deformities which had been fed the 'McCollum' diet and five with normal teeth which had received the Sherman diet. The upper incisors and lower incisors were pooled for each diet and the enamel separated from the dentine by the centrifugal flotation method (Manly and Hodge, in press). Fluorine anlyses were made on these samples in duplicate or triplicate by Dr. W. D. Armstrong of the University of Minnesota using his own method (Armstrong, '36). The results are given in table 1. The normal values are similar to those recently reported by various workers, viz., about 0.01% fluorine (Bowes and Murray, '35; Gassmann, '08; Muñoz, '36). If the estimate of Ellis and Maynard ('36) is accepted, then the fluorine content of the

[2] It should be remembered that although the diet used is designated 'McCollum' such fluorine contamination of the casein as we have described makes the diet pathological, an effect never observed by its author. For this reason, we have throughout the paper put the title McCollum in quotation marks, to signify the diet plus its contamination. Dr. E. V. McCollum was kind enough to read our manuscript before publication. The casein used in his stock breeding diet is not obtained from the same source as that used by us, and he states "In no instance have we seen any evidence of fluorosis in our stock animals. There is no discolouration of the teeth and no abnormal tooth structure such as is characteristic of fluorosis."

teeth of 'normal' rats results from the ingestion of a diet containing 3 parts per million of fluorine. The fluorine content for the dentine and enamel of the fluoride poisoned rats is suprisingly high, in fact, ten to twenty-five times the normal content and about twice the values reported by Muñoz for fluoride poisoned lower incisors.

TABLE 1

*Fluorine content of normal and fluoride poisoned incisors (data by Dr. W. D. Armstrong)*

| Incisor | Tissue | NORMAL Per cent fluorine | FLUOROSIS Per cent fluorine |
|---|---|---|---|
| Upper | | | 0.143 |
| | Enamel | 0.0079 | 0.135 |
| | Dentine | 0.0107 | 0.264[1] |
| | | 0.0108 | 0.251 |
| | | | 0.253 |
| Lower | Enamel | 0.0087 | 0.117 |
| | | 0.0083 | |
| | Dentine | 0.0111 | 0.257 |
| | | 0.0111 | 0.264 |
| | | | 0.252[2] |

[1] Average of two titrations at different times.
[2] Double distillation.

It should be noted in this table that the fluorine content of the dentine is higher than that of the enamel in both normal and abnormal teeth and is higher in approximately the same proportion in both groups.

## MEASUREMENTS OF THE LENGTH OF THE TEETH

The skulls of a total of 130 rats of different ages and from three succeding generations, taken in approximately equal numbers from the four rooms, were cleaned and mounted. Of these eleven were the skulls of normals rats fed the Sherman B diet; the remainder, the skulls of abnormal rats fed the 'McCollum' diet.

Throughout our measurements the eleven Sherman B rats have been used as the basis of comparison with the groups

taken from the different rooms. In the accompanying figures 3 and 4 the results for room I (dark room) and room III (visible room) have been plotted separately. Rooms II and IV near and far infra red rooms) have been combined.

The lengths of both upper and lower incisors were measured in the following way. The skull, magnified thirteen times, was projected by means of an opaque projector on to a screen. The curvature of the tooth was then fitted to its appropriate circle on polar coordinate paper. From the observed degree

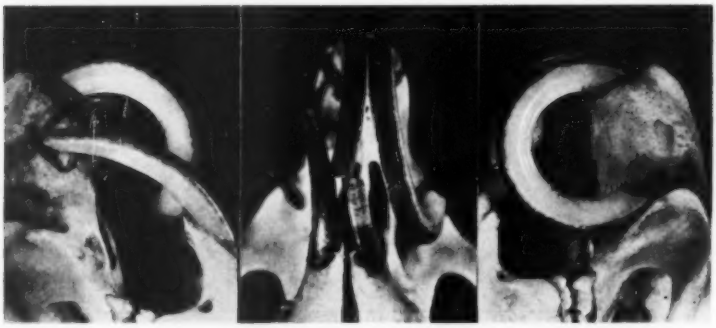

Fig. 2 A single skull shows the two principal tooth length abnormalities observed in fluorosis. On the left, an extreme overgrowth of the lower incisor has produced severe trauma of the mandibular bone. On the right is shown the more typical overgowth of the upper incisor. The center shows the right upper incisor in apposition with the left lower. The striae in the enamel are typical of fluorine.

subtended by the arc of the circle covered by the tooth the length was calculated by the following formula:

$$L = \frac{\pi R D}{180} = 0.01745 \ R \ D \text{ mm.},$$

where R = radius and D = the number of degrees. The result was corrected for magnification.

We have expressed the results of these measurements in terms of the ratio between the length of lower incisors to the length of upper incisors on the right and the left side. Normally these ratios are fairly constant with age. Donald-

son's figure expressed in this ratio is 1.3. Our results in normal rats follow his figures very closely. We have therefore taken this ratio of 1.3 as being normal.

Figure 3 shows the ratios obtained in the four rooms in rats fed on the 'McCollum' as compared with those on the Sherman B diet. A ratio markedly lower than normal except in the youngest radiated rats is at once apparent. This is due to a relative overgrowth of the upper incisors. In this series we have excluded four rats whose teeth showed such marked deformities as would render the ratios meaningless.

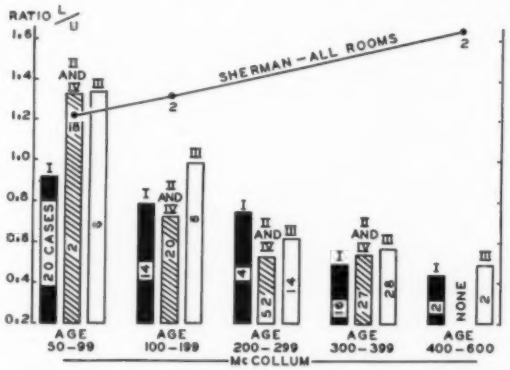


Fig. 3 Measurements of length of teeth. Sherman, normal diet; 'McCollum,' diet plus fluorine; III, animals exposed to visible radiation; II and IV, animals exposed to near and far infra-red radiation; I, animals kept in darkness.

Such an example is shown in figure 2. In this rat the right upper incisor is in apposition to the left lower, with the results that the left upper and right lower incisors show unimpeded growth. With the exception of these four rats there was good enough apposition between right and left upper and lower incisors to justify expressing the results in terms of this ratio.

*Differences in ratios possibly due to radiation.* A comparison of the ratios for length of teeth figure 3 indicates the possibility that the presence of fluorosis in the rats receiving radiation has in some way made evident, in the early stages

of the disease, some effect of light. We shall discuss this more fully in a later paper. It should, however, be noted that normal ratios were obtained in all our radiated rats in the earliest age group and that those receiving no radiation in the same age group showed the effect of fluorosis with lowered ratios. We have, it is true, fewer observations in the radiated than in the non-radiated group, so we cannot at this stage emphasize the significance of this observation.

### MEASUREMENTS OF THE ERUPTION RATE OF TEETH IN RATS FED ON THE TWO DIETS

Weekly measurements of the rate of growth of the teeth of young rats fed on both diets were kindly undertaken by Dr. B. J. Frey after we had discovered the appearance of fluorosis in the colony. His results are of such great interest that they will be published by him in detail in a separate paper. These results, where the rate of growth of the teeth is plotted against time show a remarkably slower rate of growth in the teeth of the rats fed the 'McCollum,' as compared with those fed the Sherman B diet, which confirms the preliminary findings of Smith and Lantz ('35). Doctor Frey found that the divergence in the rate of growth on the two diets is more marked in the upper than in the lower incisors, though quite definite in both. Smith and Lantz have described a lower phosphatase content in the incisors of fluoride-fed rats. It would be of considerable theoretical interest to compare the phosphatase contents of the upper and the lower incisors of normal and fluoride fed rats, in view of the markedly decreased eruption rate for the upper incisors and the slightly decreased rate for the lowers in fluorosis.

### ESTIMATION OF THE DEGREE OF COLOR OF THE TEETH

To measure the degree of color of the teeth we used a somewhat rough and arbitrary method. We employed an artist to paint, on glazed paper, degrees of color ranging from the normal, a red yellow, to the most bleached, almost

white, teeth in our group and to connect these two extremes by gradations of color.

In this way eight units of varying color strength or chroma were obtained. The Munsell values measured on the Bausch and Lomb color analyzer were obtained through the kindness of Mr. J. W. Forrest of the Bausch and Lomb Company. The Munsell values for the eight units are as follows:

| *Unit* | *Munsell Number* | *Unit* | *Munsell Number* |
|---|---|---|---|
| 8 | 6YR $\frac{6.7}{9.0}$ | 4 | 1Y $\frac{8.4}{5.0}$ |
| 7 | 8YR $\frac{7.2}{8.0}$ | 3 | 2Y $\frac{8.6}{4.6}$ |
| 6 | 1Y $\frac{8.2}{7.0}$ | 2 | 2Y $\frac{8.9}{2.5}$ |
| 5 | 10Y $\frac{8.2}{6.2}$ | 1 | 3Y $\frac{9.1}{1.5}$ |

Squares of these colors were mounted on stiff paper in series; an oblong hole 3 mm. wide was cut in the center of each to fit the upper incisors. Examination of the teeth was made in a dark room under a diffusing lamp such as is used for the examination of x-ray films. To obtain finer discrimination of shade, we examined the teeth through a Wratten (variant) filter no. 47. We measured only the upper incisors and tried as far as possible to measure the color in the middle third of the tooth.

The results of these measurements are shown in figure 4 where the age of the rat is plotted against the color of the teeth in units of chroma. These results show no variation with age in the color of the teeth of normal rats within the age limits observed. There is a marked discrepancy of color between the normal as compared with the abnormal teeth amounting to between 2 and 3 units of chroma. Perhaps more interesting still is the color of the teeth of the rats kept in the dark room on the 'McCollum' diet as compared with that of the radiated rats which were fed the same diet. At the age of 54 days (fig. 4) the dark room rats show very little color (3+ units) while at the same age the rats in room III

have an appreciable amount of pigmentation (7+ units). The rats in rooms II and IV fall intermediate at 54 days, but later the curve approximates to that of room III. All the measurements at later ages of the dark room rats are at a lower level than those of the radiated animals. This result is extremely consistent and was apparent to us on naked eye observation (color photograph).

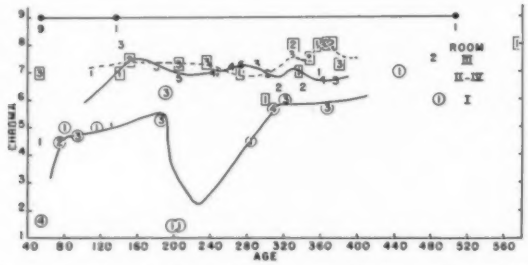


Fig. 4 Measurements of color of teeth. Upper line, rats on Sherman diet (normal); III, rats on 'McCollum' diet plus visible radiation; II and IV, rats on 'McCollum' diet plus near and far infra-red radiation; I, rats on 'McCollum' kept in darkness.

## DISCUSSION OF RESULTS

Both McCollum ('25) and Smith and Lantz ('33) noted the change in color of the teeth and recognized it as an early symptom of fluorosis. The bleaching of the teeth, as seen in our dark room rats, occurred before any signs of deformity. In the radiated rats, even at 54 days, the color of the teeth is not normal. The possibility that the presence of fluorine in one of these diets, by providing an additional physiological stress, has intensified the effects of the lack of radiation in the affected animals, has been indicated in our studies on length ratios and color of the teeth.

Fig. 5 Tooth color in 54-day-old rats. Uniform colors in the upper and in the lower incisors of the rats fed the Sherman diet (left) are obtained in the dark (room I), in near infra-red radiation (room II) or in visible light (room III). Fluorosis ('McCollum' diet) produces a lack of pigmentation in both uppers and lowers which is most evident in the dark, intermediate in near infra-red radiation and more nearly normal in visible light. The shapes of the incisors are normal in every case at 54 days.

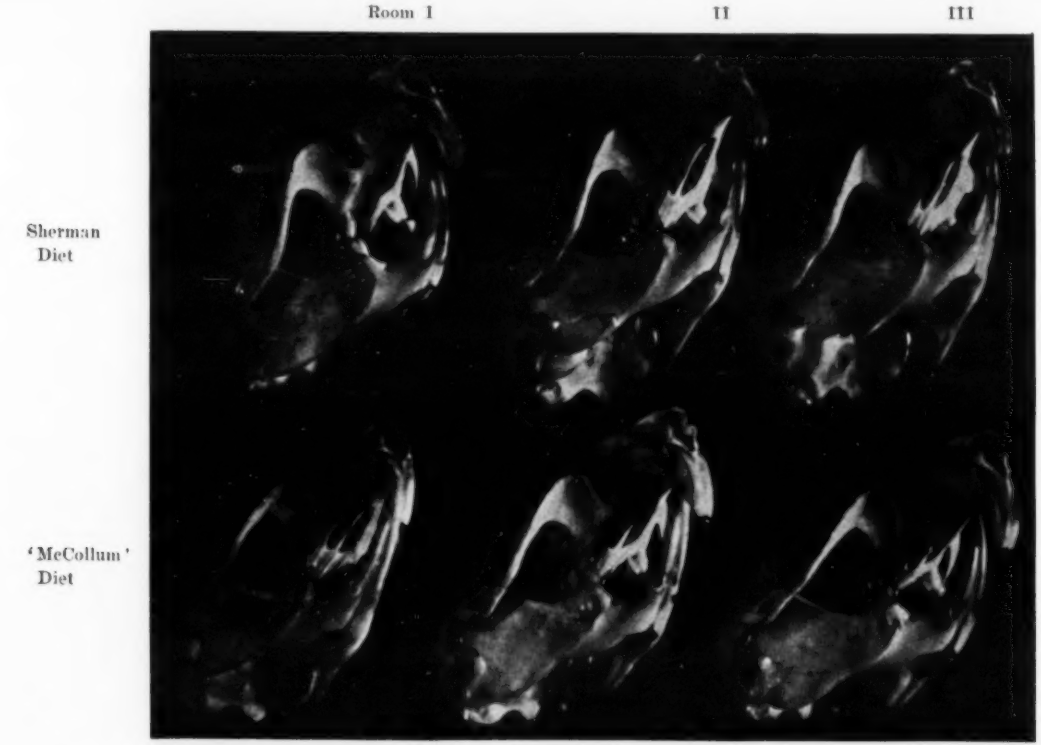


Figure 5

Little seems to be known about the physiology of pigment in the teeth of rats, nor as far as we can ascertain have the pigments present been identified. A suggestive fact in connection with light and color of teeth is the observation of Smith and Smith ('32) that in mottled enamel of human teeth, due to fluorine ingestion, the deposition of pigment is a secondary phenomenon and only appears on the lip line, or the part of the tooth exposed to light. Ellis and Maynard ('36) regard analysis for fluorine as a more sensitive test for slight degrees of fluorosis than pigment changes in teeth. It may be that in the absence of light, early pigment changes would be more apparent than in experiments conducted under ordinary laboratory conditions of light.

The source of contamination of the casein used in these experiments is unknown to us. We have no reason to suspect that such a contamination of commercial casein is of common occurrence. For though without any doubt it did occur in the casein used during the period of our experiment, yet we have recently (November, 1938) examined samples of casein, both commercial and purified, from several companies including the one from which we obtained our casein, and found them free of fluorine (on spectroscopic examination).

Our chief interest in writing up this work was to study the possible effects of radiation observed in animals fed the fluorine-contaminated diet.

The authors acknowledge with pleasure the assistance of the following persons: Dr. E. V. McCollum for reading the manuscript and for helpful criticism; Dr. L. T. Steadman for the spectrographic analyses; Dr. G. H. Ellis for the chemical analyses of the casein; Dr. W. D. Armstrong for the chemical analyses of the enamel and dentine samples; Dr. G. B. Van Schaak for mathematical study of the incisor geometry; Dr. B. J. Frey for measurements of eruption rate of the incisors, and Mr. J. W. Forrest for the Munsell values of the color comparator.

## CONCLUSIONS

1. The occurrence of fluorosis in the teeth of rats due to a contamination with fluorine of commercial casein used in an otherwise standard diet, has been described.

2. Spectroscopic analysis of the casein showed approximately 0.1% fluorine.

3. Chemical analysis of the casein showed 0.2% fluorine.

4. Chemical anlysis for fluorine of the incisor teeth of the affected rats showed a concentration which was ten to twenty-five times the normal content.

5. Measurements of length of teeth showed a great increase of upper incisors as compared with normal.

6. Measurements of the color of the teeth showed marked diminution, as compared with normal, in all affected animals.

7. The eruption rate of the teeth of the affected rats was markedly slower than normal.

8. The measurements of length and color of the teeth gave some evidence that the presence of fluorine in the diet intensified the effects of lack of radiation in the affected animals.

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# THE EFFECT OF PROTEIN AND EXERCISE AT DIFFERENT AGES ON THE TOTAL ENERGY METABOLISM

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THREE FIGURES

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This investigation was undertaken to study over long periods of time and under normal conditions of living and activity the total or over-all metabolism of experimental animals. It is the total energy metabolism, not basal, which determines food requirement and presumably is related to endurance in the broader sense, to resistance to disease, and possibly to longevity of the individual.

The specific problem was to study the effect of protein and exercise with advancing age of the experimental animal on the total energy metabolism. The source of protein as well as the level were considered by incorporating in rations two widely used proteins at an 8% and a 20% level. The two proteins chosen were supplied by whole egg and whole milk, chosen because this department in previous studies by Sumner ('38) and Sumner and Murlin ('38) had established their true digestibility and biological values in both rat and man.

The interest in this work was augmented by the program of studies being pursued by McCay, Maynard and associates (McCay, Crowell and Maynard, '35; McCay, Maynard and Sperling, '38) on the influence of various nutritional factors

[1] The data in this paper are taken from a thesis presented in partial fulfillment of the requirement for the doctorate of philosophy, University of Rochester, June, 1938.

on the length of life of the experimental animal. The problem of especial interest in these studies was the effect of protein and exercise on the total life span of rats (private communication).

## PLAN OF EXPERIMENT

The experimental subjects were ninety-six albino rats selected in groups of three, each group being matched as to sex, litter, age and weight. The experiment started shortly after the rats were weaned.

One-half of the rats or sixteen of the matched triplets were fed rations in which the protein was supplied by whole milk powder, while the other half of the animals received rations in which the protein was supplied by dried whole egg.

Within each triplet, two of the animals received a 20% protein ration and the third received a ration containing only 8% protein. One-half of the rats on the high protein ration were exercised daily to subdivide this group further.

The paired-feeding method was used as modified to include three animals in a group instead of two. The feed intake for all three was determined by the poorer eater of the two non-exercised animals. All diets from each protein were equicaloric. Those from milk and egg differed only slightly.

During the course of the experiment some difficulty was encountered in keeping all three animals of a triplet on the same food consumption and slight modifications were made at times to the rule. However, only one case occurred where the feed consumption was seriously out of balance and in this case the triplet was dropped before the end of the experiment.

The rations in which the protein was supplied by whole milk powder were composed as follows:

| | |
|---|---|
| Cellu flour | 4% |
| Sodium chloride | 1% |
| Osborne & Mendel salt mixture | 1 to 3% |
| Dried brewer's yeast | 4 to 8% |
| Cod liver oil | 1 to 2% |
| Whole milk powder[3] | 16.65 to 71.38% |
| Cornstarch | 15.62 to 48.74% |
| Crisco | 0.00 to 16.61% |

[3] Klim.

The rations in which the protein was supplied by dried whole egg were composed as follows:

| | |
|---|---|
| Cellu flour | 4% |
| Sodium chloride | 1% |
| Osborne & Mendel salt mixture | 3% |
| Dried brewer's yeast | 6 to 8% |
| Cod liver oil | 1 to 2% |
| Dried whole egg [1] | 9.26 to 38.53% |
| Cornstarch | 42.30 to 56.56% |
| Crisco | 3.67 to 17.40% |

[1] Veritas brand, purchased from Jaburg Bros., New York. Not an imported product.

As enough food to last the entire experiment could not be prepared at one time, a new lot of each ration was prepared as needed. Certain rats on the egg rations became very poor eaters after the experiment had been in progress about 105 days, indicating that there possibly might not be an optimum amount of vitamin B complex present. Additional yeast was given aside from the rations at this time to all the rats on the egg rations and when the last lot of rations was mixed, the yeast content was increased from 6 to 8% to avoid extra weighings. At this time the yeast content of the milk rations also was increased from 4 to 8% to prevent a great difference in the yeast consumption between the rats on the two rations.

The rats were exercised daily in a wooden barrel held in a horizontal position and revolved by means of an electric motor. A window was constructed in one end of the barrel so that one could be certain the rats were running and not simply sliding around. A means was also provided to ventilate the barrel while a group of rats were being exercised. Exercise on calorimeter days always preceded the heat measurement by at least 3 hours.

The program of exercising was started during the third and fourth week and continued throughout the experiment. Only a moderate amount of exercise was imposed at the start but was increased as the experiment progressed. The rats were running a distance in the barrel at the end of the first month of exercising equivalent to 997 feet, and 25 days later a dis-

tance of 1661 feet. No further change was made until the experiment had been in progress 196 days, at which time the exercise was increased to 2325 feet daily. This amount of exercise was reasonably severe since it was equivalent to a steady run of 35 minutes.

After the experiment had been in progress approximately 6 months, the number of animals in each group was reduced from sixteen to twelve as the animals were much larger and a smaller number could, therefore, be used satisfactorily in the

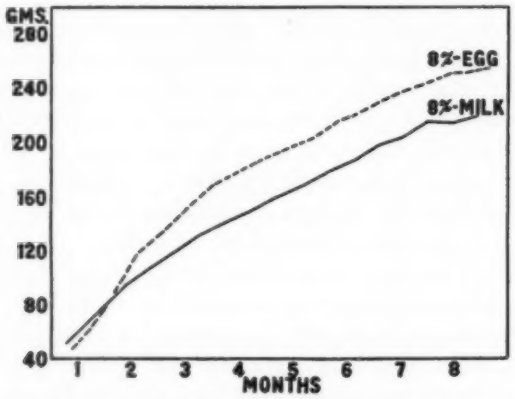


Fig. 1 Average growth of rats with the same energy intake but different source of protein.

calorimeter. The reduction was made so as to leave in each group an equal distribution of sex of as uniform weight as possible. Obviously all three animals of a triplet were either retained or discarded.

The heat production was measured by means of a new automatic calorimeter which has been described recently by Barrows and Murlin ('38). A group of twelve to sixteen rats was placed in the calorimeter in small individual cages at one time and the heat production was measured continuously for 19 to 22 hours by both direct and indirect methods. Each rat had a

day's food supply and water and was permitted to move around within the limits of the individual cage at will.

The apparatus gave theoretical values for heat as well as for the oxygen, carbon dioxide and R.Q. of alcohol. The data are omitted in the interest of economy of space.

## RESULTS

The growth curves of the twelve rats from each of the six groups that were kept for the entire experiment are shown in figures 1 and 2. An equal distribution of sexes is represented in all the curves.

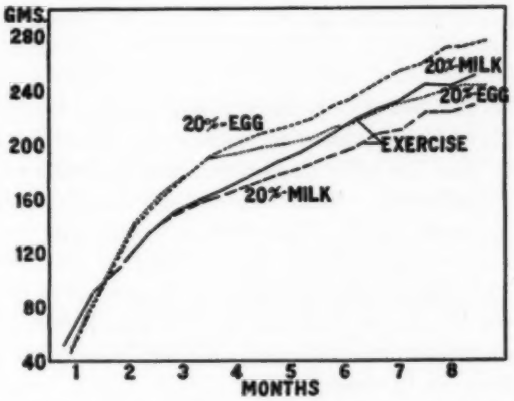


Fig. 2 Average growth of rats with the same energy intake but different source of protein.

A comparison of the growth promoting qualities of the proteins of two such important and widely used foods as egg and milk is of considerable practical value. The three groups of rats on the milk rations gained on the average 199, 168 and 176 gm. for the 20% protein, 8% protein and 20% protein and exercise groups respectively; while the gain on the egg rations was 229, 207 and 195 gm. for the 20% protein, 8% protein and 20% protein and exercise groups respectively. The gain of the group on the 20% egg protein ration of 229 gm. is a little

high since one female rat of this group died before the end of the experiment. If the last weight taken be considered as the final weight for the rat that died, then the average gain of 229 gm. is 6 gm. high. The odds expressing the degree of significance of the gains in body weight, computed by the modification of Love ('24) of the method of 'Student' ('08) were as follows:

| | *Milk rations* | *Egg rations* |
|---|---|---|
| Gain in weight of 20% protein over 8% protein | 10,000: 1 | 195: 1 |
| Gain in weight of 20% protein over 20% protein with exercise | 3,332: 1 | 3,332: 1 |
| Gain in weight of 20% protein with exercise over 8% protein | 19: 1 | ..... |
| Gain in weight of 8% protein over 20% protein with exercise | ..... | 908: 1 |

The low protein group on the egg ration made a rather remarkable gain, approaching sufficiently near the high protein group that the odds (195: 1) do not indicate a difference of great significance. This was not true on the milk rations as the gain made by the high protein group was very significantly greater (odds 10,000 to 1) than by the low protein group. The superiority of egg protein over that of milk was best shown by comparison of the low egg protein with that of the high milk protein, the gain on the former being slightly greater than on the latter.

The inclusion of the two exercised groups affords a comparison not usually made in nutrition studies, namely to test the efficiency of the rations for producing work. The growth curves show that early in the study when little exercise was given the group on the egg ration gained weight faster than the group on the milk ration. This was to be expected, but when more exercise was given the difference was not so marked, and the growth curves for the two exercised groups tended to become closer as the experiment progressed. On the milk rations the low protein group gained less than the exercised group throughout, but on the egg rations the low protein group gained more than the exercised group, though

only in the last 2 months. The difference was slight in the case of the groups receiving milk rations but quite significant at the end in the case of the groups receiving egg rations. From the foregoing comparisons there appears to have been a greater expenditure of energy by the group receiving the egg ration than by the group receiving the milk ration for producing an equal amount of work.

The greater gain made by the high protein groups, which were not exercised, was apparently due to a larger deposition of fat as judged by the amount of subcutaneous fat.

As the amount of feed given the rats on the milk rations was determined independently from the amount given those on the egg rations, it was not expected the total intake of feed for all six groups would be approximately the same at the end of the experiment, yet this proved to be the case. This coincidence is of considerable value in that all six groups can be compared on the basis of approximately equal energy intakes. The total nitrogen intake of the four groups on high protein rations also was very similar. This information is summarized in table 1.

TABLE 1

*Total nitrogen and energy intake by groups for the entire experiment*

| *Group* | *Nitrogen gm.* | *Energy Calories* |
|---|---|---|
| 20% protein-milk | 901.53 | 138,971.9 |
| 8% protein-milk | 368.06 | 140,302.1 |
| 20% protein-milk with exercise | 899.22 | 138,611.0 |
| 20% protein-egg | 897.16 | 136,591.8 |
| 8% protein-egg | 373.93 | 136,087.1 |
| 20% protein-egg with exercise | 904.67 | 137,763.2 |

All of the egg rations produced greater growth than the corresponding milk rations notwithstanding that some less energy was consumed on the former. The significance of the results is increased by a difference of this nature. Errors which may be expected to occur in chemical analyses alone are sufficient to account for most of the differences in nitrogen and energy intakes between the various groups.

The heat production values presented in table 2 are the averages of the direct and indirect heat determinations for an equal number of periods equally distributed for each group. The lengths of the periods are 3 hours for period 1 and 16 hours for period 2; and in cases where there are periods 1, 2 and 3, the first two are of 3 hours duration each and the third of 16 hours duration. The surface area was computed by the use of Diack's ('30) formula.

There is an average difference between the direct and indirect heat measurement in eighty-one separate periods of 4.9%, with a standard deviation of $3.6 \pm 0.19$. The agreement between the direct and indirect heat measurement is quite as close with this apparatus as with the conventional type of calorimeter; besides the ease of operation is remarkably greater, as an operator must be present only a very small amount of the time. The deviation between the two methods of measuring the heat was not always in the same direction; the directly determined heat was higher than the indirect approximately as many times as it was lower.

The average total heat production for the experiment for all groups of animals with standard deviations is given in table 3. In figure 3, the heat production is plotted according to increasing age of the experimental animals.

As shown in figure 3, the heat production of all six groups of rats decreased with increasing age for about 5 to 6 months. A decrease in basal metabolism with increasing age is clearly established for most species of animals. As the basal constitutes such a large part of the total metabolism, the latter would be expected to follow generally the trend of basal metabolism.

The rise in total metabolism after a rather long period of decline was an unexpected finding. This increase occurred earlier in the two high protein groups on the egg rations than in any other groups. The high point on the curve of heat production for the sixth month for the 20% protein group from eggs cannot reasonably be attributed to a poor heat determination as there was good agreement between the two

TABLE 3

*Summary of heat production determinations*

| GROUP | AGE OF RATS | PERIOD | HEAT PRODUCTION PER SQUARE METER OF SURFACE | GROUP | AGE OF RATS | PERIOD | HEAT PRODUCTION PER SQUARE METER OF SURFACE | GROUP | AGE OF RATS | PERIOD | HEAT PRODUCTION PER SQUARE METER OF SURFACE |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 20% protein from milk | 59 | 1 | 44.4 | 20% protein from milk exercise | 63 | 1 | 41.8 | 8% protein from eggs | 63 | 1 | 49.4 |
| 20% protein from milk | 59 | 2 | 45.6 | 20% protein from milk exercise | 63 | 2 | 45.9 | 8% protein from eggs | 63 | 2 | 50.3 |
| 20% protein from milk | 143 | 1 | 41.9 | 20% protein from milk exercise | 147 | 1 | 44.0 | 8% protein from eggs | 149 | 1 | 37.2 |
| 20% protein from milk | 143 | 2 | 44.2 | 20% protein from milk exercise | 147 | 2 | 42.0 | 8% protein from eggs | 149 | 2 | 41.2 |
| 20% protein from milk | 171 | 1 | 41.3 | 20% protein from milk exercise | 161 | 1 | 43.1 | 8% protein from eggs | 163 | 1 | 36.8 |
| 20% protein from milk | 171 | 2 | 43.7 | 20% protein from milk exercise | 161 | 2 | 40.8 | 8% protein from eggs | 163 | 2 | 39.8 |
| 20% protein from milk | 199 | 1 | 39.0 | 20% protein from milk exercise | 203 | 1 | 42.1 | 8% protein from eggs | 205 | 1 | 37.9 |
| 20% protein from milk | 199 | 2 | 39.2 | 20% protein from milk exercise | 203 | 2 | 41.6 | 8% protein from eggs | 205 | 2 | 35.8 |
| 20% protein from milk | 199 | 3 | 42.7 | 20% protein from milk exercise | 203 | 3 | 41.0 | 8% protein from eggs | 205 | 3 | 38.7 |
| 20% protein from milk | 248 | 1 | 43.2 | 20% protein from milk exercise | 252 | 1 | 44.0 | 8% protein from eggs | 254 | 1 | 39.1 |
| 20% protein from milk | 248 | 2 | 44.1 | 20% protein from milk exercise | 252 | 2 | 43.0 | 8% protein from eggs | 254 | 2 | 38.2 |
| 20% protein from milk | 248 | 3 | 46.6 | 20% protein from milk exercise | 252 | 3 | 46.0 | 8% protein from eggs | 254 | 3 | 43.3 |
| 8% protein from milk | 61 | 1 | 42.8 | 20% protein from eggs | 64 | 1 | 45.0 | 20% protein from eggs exercise | 67 | 1 | 43.0 |
| 8% protein from milk | 61 | 2 | 53.4 | 20% protein from eggs | 64 | 2 | 45.9 | 20% protein from eggs exercise | 67 | 2 | 47.8 |
| 8% protein from milk | 145 | 1 | 41.6 | 20% protein from eggs | 147 | 1 | 38.7 | 20% protein from eggs exercise | 151 | 1 | 44.1 |
| 8% protein from milk | 145 | 2 | 48.1 | 20% protein from eggs | 147 | 2 | 38.6 | 20% protein from eggs exercise | 151 | 2 | 39.7 |
| 8% protein from milk | 158 | 1 | 38.3 | 20% protein from eggs | 161 | 1 | 37.3 | 20% protein from eggs exercise | 165 | 1 | 42.0 |
| 8% protein from milk | 158 | 2 | 43.8 | 20% protein from eggs | 161 | 2 | 39.3 | 20% protein from eggs exercise | 165 | 2 | 37.5 |
| 8% protein from milk | 201 | 1 | 37.8 | 20% protein from eggs | 203 | 1 | 37.9 | 20% protein from eggs exercise | 207 | 1 | 44.4 |
| 8% protein from milk | 201 | 2 | 36.0 | 20% protein from eggs | 203 | 2 | 37.2 | 20% protein from eggs exercise | 207 | 2 | 41.7 |
| 8% protein from milk | 201 | 3 | 43.9 | 20% protein from eggs | 203 | 3 | 39.3 | 20% protein from eggs exercise | 207 | 3 | 41.6 |
| 8% protein from milk | 250 | 1 | 40.3 | 20% protein from eggs | 252 | 1 | 39.2 | 20% protein from eggs exercise | 256 | 1 | 44.2 |
| 8% protein from milk | 250 | 2 | 44.9 | 20% protein from eggs | 252 | 2 | 37.7 | 20% protein from eggs exercise | 256 | 2 | 45.1 |
| 8% protein from milk | 250 | 3 | 44.0 | 20% protein from eggs | 252 | 3 | 41.7 | 20% protein from eggs exercise | 256 | 3 | 43.6 |

methods of determining the heat production. An unusual amount of activity must have occurred during this calorimeter determination to have increased the heat production by such an amount.

The increased heat production which occurred in the latter part of the experiment was apparently caused by a number of factors each contributing a part. Some of the factors which may reasonably be supposed to have influenced the heat production are the following:

The growth impulse in the rat is tremendous. A good example of this is the ability of the rat to grow considerably after 2 or 3 years of retardation (McCay, Crowell and Maynard, '35). As the paired-feeding method does not permit

TABLE 3

*Average heat production*[1] *for equal number of determination on each group*

| GROUP | MILK RATIONS | | EGG RATIONS | |
|---|---|---|---|---|
| | Average age | Heat production | Average age | Heat production |
| | *days* | *Cal./sq.m./hr.* | *days* | *Cal./sq.m./hr.* |
| 20% protein | 164 | 43.0, $\sigma = 2.2$ | 165 | 39.8, $\sigma = 2.8$ |
| 8% protein | 163 | 42.9, $\sigma = 3.3$ | 167 | 40.6, $\sigma = 3.8$ |
| 20% protein + exercise | 165 | 42.9, $\sigma = 1.7$ | 169 | 42.9, $\sigma = 2.5$ |

[1] Values shown are based on the same number of long and short periods, equally distributed as to age.

the maximum growth rate, the rat can reasonably be expected to be less wasteful of energy until the growth impulse is satisfied. Thereafter more energy remains unclaimed for retention which would appear as dynamic effect.

Another circumstance to help explain the increased heat production as found would be an increase in activity. There is much less inclination and need for a mature animal to sleep than one that is young and growing rapidly. The total activity may be roughly proportional to the amount of time spent awake.

The activity may also have been increased by thwarted sexual impulse which must be strong in rats from 5 months

upward especially as both sexes were in small individual cages in the calorimeter at one time.

Another point shown by the curve of heat production is that the low protein groups on both the egg and milk rations had a higher heat production at the start of the experiment, lasting to beyond 4 months on the milk ration, than the high protein groups. This confirms the work of Forbes, Swift, Black and Kahlenberg ('35) in which a higher heat production was found on a 10% protein ration than any protein level up to 25% in experiments of 10 weeks of duration. But in

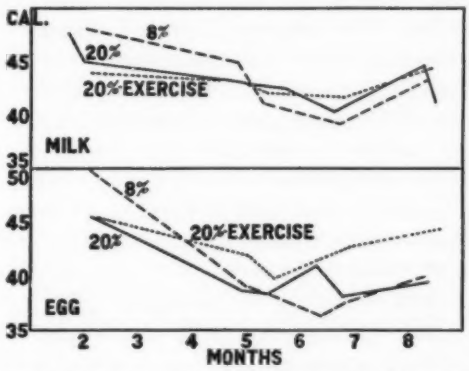


Fig. 3 Total heat production with increasing age as affected by differences in source and amount of protein intake and by exercise. All heat measurements that were made are included in this figure.

the present investigation the lowest heat productions also were obtained later in the experiment, when the animals were older, with the same low protein groups.

There was less variation as regards heat production between the two exercised groups than between any other two groups. As these animals did not get all they could eat and were exercised uniformly each day, the total activity would not be expected to vary so much throughout the experiment.

The average heat production of an equal number of periods equally distributed for the entire experiment was essentially the same for the three groups on the milk rations as shown

in table 3. The exercised group on the egg ration had a higher average heat production than the two other groups on the egg rations that were not exercised. This difference was accumulated throughout most of the experiment, as the heat production curve (fig. 3) was highest for the exercised group at all times except early in the experiment when little exercise was given. The exercised group converted less feed energy into body gain than did the other two groups on the egg rations.

On the basis of the average heat production values for the experiment, the level of protein intake did not influence the heat production. The source of protein did affect slightly the heat, as there was a lower production on the egg rations than on the milk rations on both the high and low intake, except in groups of the experimental animals which were exercised. When protein is retained for growth of muscle or other tissue, it is withdrawn from dynamic effect. Milk does not produce tissue so well, and it appears to cause a greater specific dynamic effect with consequent higher heat production. However, these differences prove not to be statistically significant. Thus in table 3 the means for 20% milk and egg are 42.4 and 39.8 Cal./square meter/hr. respectively. This difference between means of 2.6 Cal. is not greater than the square root of the sum of the squares of the two standard errors of the means. Significance requires that the difference be two times (Davenport and Ekas, '36) this square root.

## SUMMARY

The effects of the proteins of whole milk powder and dried whole egg, incorporated in equicaloric rations at both a high and a low level (8 and 20%), were studied on the total energy metabolism of rats from weanling to 8½ months of age with a direct-indirect automatic calorimeter. The effects of a mild amount of exercise were also studied in one-half of the animals on the high protein ration. Feeding was by the paired method as modified to include three animals on the same protein in a group.

A marked superiority of the protein of egg over milk for producing growth was shown when the energy intake was the same.

The total heat production of all six groups of animals, expressed on a surface area basis, decreased rather decidedly with increasing age during the first 5 to 6 months of the experiment. After this an increase in heat production was found in all groups which continued at an increasing rate until the end of the experiment. Possible reasons for this are proposed.

The average total metabolism for the entire experiment was essentially the same for the three groups on the milk rations. It was lower for the two non-exercised groups on the egg rations than for the exercised groups.

The level of protein did not influence the average total heat production.

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# THE EFFECT OF PROTEIN AND EXERCISE AT DIFFERENT AGES ON THE BASAL METABOLISM [1]

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The investigation to be discussed is a part of a previous study of the effect of protein and exercise on the total energy metabolism reported in this issue of this Journal (Black and Murlin, '39).

In this study equicaloric rations containing 8 and 20% protein from both whole egg and whole milk were fed to rats from weanling to 8½ months of age. The rats on the high protein rations were divided into two groups, one group receiving a moderate amount of exercise daily. The three groups of animals receiving either source of protein were selected in triplets. Each animal of a triplet was of the same sex, litter, age and weight and was given the same amount of food.

During the seventh to the eighth month of the experiment, basal metabolism determinations were made on a representative number of animals from each group.

The results of the total energy metabolism investigations together with a full description of the experimental procedure followed in the treatment of the animals is given in the previous paper.

The study of the basal metabolism of rats much older than those just described was made possible through the cooperation of the Laboratory of Animal Nutrition of Cornell Uni-

[1] Part of a thesis presented in partial fulfillment of the requirement for the doctorate of philosophy, University of Rochester, June, 1938.

versity. The investigation dealing with total energy metabolism was planned so that comparisons in the energy metabolism of the animals in this experiment could be made with those in the study of the effect of protein and exercise on the length of life of rats which was being conducted by McCay and Maynard at Cornell. Since these workers were not equipped to study energy metabolism, they very kindly relinquished that phase of the investigation to this laboratory.

By transporting the metabolism apparatus to the Cornell Laboratory, basal metabolism determinations were made on a number of very old rats which had received comparable treatment to those used in this department.

The apparatus used for basal metabolism determinations was of the Haldane principle; moisture and carbon dioxide production and the oxygen consumption were determined gravimetrically. The particular form of apparatus used was constructed similarly to the one described by Forbes, Kriss and Miller ('34), with a few minor modifications.

All measurements were made with the respiration chamber maintained at a temperature between 28°C. and 29°C. The length of the experimental periods was from 6 to 7 hours. The carbon dioxide production for the first hour, and occasionally the second hour, was always excluded from the calculation of the heat production because of activity of the animal; however these hours were not excluded from the calculation of the respiratory quotient. The heat production was computed from the average respiratory quotient and the hourly carbon dioxide production.

The basal heat productions of the rats selected from the experiment in this department are shown in table 1. These values are expressed as calories per square meter of body surface per hour. Surface area was estimated by the use of Diack's ('30) formula. The plan followed in the selection of rats from each group for basal metabolism determinations was to use matched animals, and therefore an equal distributions of sexes was represented.

TABLE 1

*Basal heat production, rats at Rochester*

| PROTEIN SUPPLIED BY MILK | | | | | PROTEIN SUPPLIED BY EGGS | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Rat | Sex | Basal heat per hour | Surface area | Basal heat per square meter per hour | Rat | Sex | Basal heat per hour | Surface area | Basal heat per square meter per hour |
| | | *Cal.* | *sq.cm.* | *Cal.* | | | *Cal.* | *sq.cm.* | *Cal.* |
| | | 20% protein ration | | | | | 20% protein ration | | |
| 1 | M | 1.073 | 321 | 33.4 | 58 | F | 0.777 | 263 | 29.5 |
| 4 | F | 0.838 | 251 | 33.4 | 61 | F | 0.842 | 260 | 32.4 |
| 13 | M | 1.131 | 321 | 35.2 | 64 | M | 1.105 | 361 | 30.6 |
| 19 | M | 1.066 | 335 | 31.8 | 67 | F | 0.803 | 273 | 29.4 |
| 22 | M | 1.348 | 334 | 40.4 | 73 | M | 1.125 | 375 | 30.0 |
| 25 | F | 0.728 | 245 | 29.7 | 79 | M | 1.014 | 323 | 31.4 |
| 34 | F | 0.756 | 255 | 29.6 | 85 | M | 1.060 | 334 | 31.7 |
| 37 | M | 0.913 | 302 | 30.2 | Average | | 0.961 | 312 | 30.7 |
| Average | | 0.982 | 296 | 33.2 | | | | | |
| | | 8% protein ration | | | | | 8% protein ration | | |
| 2 | M | 0.872 | 296 | 29.5 | 59 | F | 0.709 | 254 | 27.9 |
| 5 | F | 0.755 | 235 | 32.1 | 62 | F | 0.809 | 255 | 31.7 |
| 14 | M | 0.942 | 310 | 30.4 | 65 | M | 1.137 | 352 | 32.3 |
| 20 | M | 0.930 | 304 | 30.6 | 68 | F | 0.790 | 265 | 29.8 |
| 23 | M | 1.001 | 303 | 33.0 | 74 | M | 1.239 | 360 | 34.4 |
| 26 | F | 0.717 | 225 | 31.9 | 80 | M | 0.973 | 306 | 31.8 |
| 35 | F | 0.685 | 228 | 30.0 | 86 | M | 1.092 | 320 | 34.1 |
| Average | | 0.845 | 272 | 31.1 | Average | | 0.964 | 302 | 31.9 |
| | | 20% protein ration and exercise | | | | | 20% protein ration and exercise | | |
| 3 | M | 1.220 | 312 | 39.1 | 60 | F | 0.830 | 250 | 33.2 |
| 6 | F | 0.850 | 237 | 35.9 | 63 | F | 0.933 | 255 | 36.6 |
| 15 | M | 1.055 | 306 | 34.5 | 66 | M | 1.226 | 341 | 36.0 |
| 21 | M | 1.099 | 293 | 37.5 | 69 | F | 0.821 | 272 | 30.2 |
| 24 | M | 0.979 | 295 | 33.2 | 81 | M | 1.080 | 292 | 37.0 |
| 27 | F | 0.708 | 230 | 30.8 | 87 | M | 1.068 | 304 | 35.1 |
| 36 | F | 0.837 | 228 | 36.7 | Average | | 0.993 | 286 | 34.7 |
| Average | | 0.964 | 272 | 35.4 | | | | | |

In table 2 the basal heat productions of the rat studied at Cornell are given as well as the age, treatment and general condition of the animals. This experiment was designed to study the effect of the proteins, casein and liver, fed at a high and low level, with and without exercise, on the total life span. All the animals in this experiment were males. The food consumption was not determined, but the animals were in general, showing a slight decrease in weight. Some loss in weight may be expected as a natural consequence in rats of this age.

The rations fed in this experiment were composed of certain constant constituents which were as follows: sucrose 10%, cellulose 2%, butter 5%, yeast (partly irradiated) 5%, salt mixture 3 to 4% and carotene at the rate of 1 mg. per kilogram of ration. In addition to these constant constituents the remainder of the four rations was composed as follows: high protein ration from casein: cooked starch 34%, casein 29%; lactalbumin 4% and lard 7%; high protein ration from liver: cooked starch 34% and dry liver 41%; low protein ration from casein: cooked starch 65%, casein 4%, lactalbumin 3% and lard 2%; low protein ration from liver: cooked starch 65% and dried liver 10%.

The high and low protein rations from casein contained approximately 27 and 7% protein respectively while the corresponding rations from liver contained 28 and 9% protein.

There was observed a wide fluctuation in the basal metabolism of the old rats that cannot be attributed to any external conditions. The animals were less active on the whole than the younger ones were during the time they were in the respiration chamber. One animal, K205, was not active but was in a state of high muscular tension as noted by one leg quivering at times. A high metabolism could therefore be expected under these circumstances.

Four rats in this group breathed with apparent difficulty; however, this peculiarity of breathing was manifest whether in the respiration chamber or the regular cage. The respirations were rather slow, very deep and seemed to require extra

TABLE 2

*Basal heat production, male rats at Cornell*

| RAT | WEIGHT | PROTEIN FED | PROTEIN LEVEL | AGE | BASAL HEAT/SQ. METER/HR. (DIACK) | CONDITION OF ANIMALS |
|---|---|---|---|---|---|---|
| | | | | *days* | *Cal.* | |
| Rats exercised | | | | | | |
| K 649 | 298 | Casein | High | 673 | 41.5 | Good |
| K 767 | 203 | Liver | High | 641 | 43.4 | Very thin, died in run |
| K 229 | 264 | Casein | High | 859 | 42.4 | Good |
| K 652 | 301 | Casein | High | 676 | 38.0 | Fair, very thin |
| K 827 | 323 | Casein | High | 636 | 39.2 | Good |
| K 396 | 302 | Liver | High | 712 | 38.1 | Fair |
| K 778 | 307 | Liver | High | 703 | 31.2 | Good |
| K 816 | 307 | Liver | High | 700 | 38.7 | Good |
| K 771 | 356 | Liver | Low | 636 | 32.9 | Lost most of hair from stomach |
| K 41 | 321 | Casein | Low | 871 | 36.1 | Good, some hair lost from stomach |
| K 831 | 333 | Casein | Low | 646 | 31.0 | Good |
| K 205 | 342 | Liver | Low | 869 | 42.7 | Fair, some hair lost from stomach, tense |
| K 224 | 333 | Liver | Low | 854 | 38.2 | Hair very thin |
| K 504 | 411 | Liver | Low | 720 | 33.1 | Good |
| K 517 | 359 | Liver | Low | 707 | 41.7 | Good |
| K 819 | 357 | Liver | Low | 649 | 32.4 | Fine |
| Average | | | | 722 | 37.5 | |
| Rats not exercised | | | | | | |
| K 391 | 328 | Liver | High | 708 | 46.4 | Good |
| K 579 | 286 | Casein | High | 714 | 41.9 | Fair, breathes hard |
| K 413 | 271 | Liver | High | 717 | 40.9 | Fair, breathes hard |
| K 522 | 301 | Liver | High | 715 | 31.0 | Fine |
| K 531 | 299 | Liver | High | 717 | 29.6 | Fine |
| K 691 | 327 | Liver | High | 682 | 38.5 | Very good |
| K 814 | 282 | Liver | High | 645 | 41.1 | Good, breathes slightly hard |
| K 480 | 276 | Casein | Low | 713 | 40.4 | Not very good, breathes hard |
| K 550 | 338 | Casein | Low | 713 | 36.1 | Good |
| K 646 | 353 | Casein | Low | 680 | 30.5 | Fine, reasonably fat |
| K 829 | 299 | Liver | Low | 641 | 30.3 | Good |
| Average | | | | 695 | 37.0 | |

effort on the part of the animal. As expected, the metabolic rate of these rats was found to be high, but no higher than in others that did not have this peculiarity in breathing.[2]

Basal metabolism determinations were made on all the rats in this experiment that were alive at the time and which did not show pathological conditions or from natural reasons appeared near death. It was not anticipated that rat K767 was so near death, or it would not have been used. The chief interest was in basal metabolism of old age under as nearly normal conditions as possible without contamination with obvious pathological conditions.

### RESULTS WITH RATS AT ROCHESTER

The high protein group on the milk rations produced on the average slightly more heat than the low protein group indicating that possibly the basal metabolism was lowered by a low protein ration. The odds are not significant (16:1) in this case as there are so few animals. The exercised group on the milk rations had the highest average heat production of any group on the milk rations, but again the odds do not indicate that this difference is significantly greater than the non-exercised high protein group. They do indicate a slight significance (odds 87:1) over the non-exercised low protein group.

The two non-exercised groups on the egg rations had nearly the same basal heat production, but the average for the low protein group was higher than for the high protein group in this case. The exercised group again had the highest basal heat production of any group on the egg ration as was the case on the milk rations. The odds that the higher basal metabolism of the exercised group (than that of the 20% protein non-exercised group), was not due to chance alone, are 666:1. The odds are 158:1 that the heat production of the exercised group was really greater than that of the low protein group.

[2] Since this paper went to press Dr. McCay has discovered that his old rats are quite subject to a low-grade pneumonia.

If the corresponding groups are combined by not considering the source of protein, the highest basal heat production occurred in the high protein group receiving exercise and the lowest in the low protein group. The high protein group which was not being exercised produced slightly more heat on the average than the low protein group. The magnitude of the difference between the exercised group and the low protein group is expressed by odds of 3332:1, and the difference between the exercised group and the high protein group by odds of 103:1.

A number of differences found in the total metabolism have also been found to occur in the basal metabolism. The average total metabolism was the same for all three groups on the milk rations. While the basal metabolism for these groups was not the same, there were no differences of especial significance. The basal metabolism of the two non-exercised groups on the egg rations was lower on the average than the two non-exercised groups on the milk rations which was also true for the total metabolism. The total metabolism of the exercised group on the egg rations was considerably higher at the end of the experiment than the two other groups on the egg rations; also the basal metabolism was higher.

The explanation for the higher basal metabolism in the exercised animals may have been due to a higher degree of muscular tonus, less subcutaneous fat, more muscular tissue or other factors.

The feed intake of the exercised animals was limited by animals that were not being exercised, and this limitation was sufficient to keep the body weight well below the group that was not exercised. It is well known that undernutrition lowers the metabolism, but in spite of any effect this factor may have had on the basal metabolism of the exercised rats, the result was a higher metabolism. This lends further support to the significance of exercise increasing the basal metabolism.

The difference between the average total metabolism during the final $2\frac{1}{2}$ months of the experiment and the basal metabo-

lism was 26%, which is called supermetabolism. This difference includes all six groups of animals. The largest supermetabolism, 32%, occurred in the low protein group on the milk ration.

The generally accepted idea that the basal heat production of female rats is lower than that of males is upheld in this work, as the average of the twenty-four male rats is 804 and of the eighteen female rats is 760 Calories per square meter of body surface per 24 hours.

### RESULTS WITH RATS AT CORNELL

Exercising of the rats in this experiment continued throughout the life of the animals until old age and was terminated approximately 5 months before the basal metabolism measurements were made. Under these conditions the exercise had not influenced the metabolism. If the prolonged program of work imposed on these animals speeded up the vital-functions of the body and, therefore, the basal metabolism and total energy expenditure, its effect was not permanent. The average of the eight rats on high protein diet which had been exercised was 39.1 Cal./sq.m./hr., while the average of the six on high protein which had not been exercised was 38.5 Cal. Also the basal metabolism of the low protein groups was very similar.

The basal heat production of the fifteen rats on the high protein intake was 38.8 Calories per square meter of body surface per hour, while it was only 35.5 Calories per hour in the twelve rats on the low protein intake. A difference of this magnitude is probably significant. In the experiment with the rats at Rochester where identical amounts of equicaloric rations of high and low protein content were fed, the basal metabolism was not altered.

The metabolism of one rat (K767) that died in the respiration chamber, was high immediately preceding death. There was also a reasonably large premortal rise.

In this experiment, the heat production was calculated by three different methods, Diack's formula, Lee's formula

and per unit of body weight. The comparative results were unaffected by the method of calculation.

In comparing the basal metabolism of the young and old rats, a comparison of animals of the same weight has a distinct advantage since it eliminates the possibility of errors which may be involved in calculating the heat production to a comparable basis. In these experiments, it was possible to select eleven of the 7- to 8-month-old rats of almost exactly the same weight as eleven of the 700-day-old rats for such a comparison. The basal metabolism of the young rats was 814 Calories and of the 700-day-old rats was 924 Calories per square meter of body surface per 24 hours. This was certainly not due to activity as the older rats appeared quieter than the younger ones. The two methods of feeding cannot be wholly reconciled as being without some influence on the metabolism. It seems reasonable to think that some of the difference noted in the basal metabolism must have been due to age. There may be good reasons for old rats to show a higher basal metabolism than younger ones. The size of the organs, the amount of subcutaneous fat and the degree of relaxation are some of the factors which may be responsible for the difference in metabolism.

## SUMMARY

The basal metabolism of rats 7 to 8 months of age was studied and compared with the total metabolism of these same animals and also with the basal metabolism of other rats 700 days of age. In the investigation involving the younger animals, the effects on metabolism of the proteins of whole milk and whole egg fed at a high and low level and of exercise were observed. With the older rats, the influence of the level of protein intake, exercise and no exercise, was studied on the length of life of the animal.

In the study involving the younger rats, the basal heat production was the lowest on the average in the low protein group, somewhat higher in the high protein group and highest in the high protein group that was being exercised. The dif-

ference in heat production between any two groups is not great except in the case of the low protein group and the high protein group receiving exercise, in which case the difference is quite significant.

The supermetabolism, or the difference between the total metabolism and the basal metabolism, was 26%.

With the old rats, a high protein intake was associated with a higher basal metabolism.

In comparisons between animals of equal weights, a higher basal metabolism was found in the older animals.

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# THE PREVENTIVE EFFECT OF WHEAT GERM OILS AND OF α-TOCOPHEROL IN NUTRITIONAL MUSCULAR DYSTROPHY OF YOUNG RATS [1]

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Rats on certain vitamin E-low diets develop nutritional muscular dystrophy. The paralytic symptoms of this disease were observed for the first time in the laboratory of Evans and Burr ('28) in the young of female rats that had been given the minimum amount of vitamin E to produce a litter. The disorder appears on or about the twentieth day of life and is characterized by severe degeneration of the skeletal muscles (Olcott, '38). Symptoms of muscle weakness have been reported in older animals maintained on vitamin E-low diets from the time of weaning (Ringsted, '35; Burr, Brown and Moseley, '37; Einarsten and Ringsted, '38; Evans, Emerson and Telford, '38; Knowlton and Hines, '38). Several of these authors have also described slight lesions in the muscles.

Evans and Burr ('28) showed that the paralysis was prevented by the administration to either mother or young during the period of lactation, of wheat germ oil or vitamin E concentrates. The protective effect of wheat germ oil and concentrates of vitamin E was confirmed by Morelle ('31), Mason ('33), Olcott and Mattill ('34) and Ringsted ('35). However, in similar experiments with diets of natural foods in which

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the anti-sterility factor had been inactivated by treatment with ethereal ferric chloride (Waddell and Steenbock, '28), young rats remained normal (Goettsch and Pappenheimer, '31).

The present paper concerns the role of vitamin E in muscle dystrophy of the young rat. A comparison has been made of the anti-sterility potency and the muscle dystrophy preventing effect of wheat germ oil, of oil of treated wheat germ in which the vitamin E has been inactivated by ferric chloride treatment, and of α-tocopherol (Evans, Emerson and Emerson, '36).

## EXPERIMENTAL

*Diets and supplements.* The diets, similar to those used by Evans and Burr ('28), had the following composition:

| | *Diet I* % | *Diet II* % |
|---|---|---|
| Casein (Merck's technical) | 29.1 | 26.6 |
| Cornstarch | 36.3 | 33.3 |
| Lard | 20.0 | 18.3 |
| Yeast, bakers' dried | 9.0 | 16.6 |
| Salt mixture (Hawk and Oser, '31) | 3.6 | 3.4 |
| Cod liver oil (Mead Johnson) | 2.0 | 1.8 |

Diet I is low in vitamin E for, in all female rats (thirty-four) reared and bred upon it, the first gestations resulted in resorptions. Diet II containing more yeast was used during the lactation period.

*Wheat germ oil.* Wheat germ was extracted in a continuous extraction apparatus with petroleum ether which had been purified by treatment with concentrated sulfuric acid for several weeks, followed by washing and distillation. The oil was used within a month of preparation.

*Oil of treated wheat germ.* Ten grams of ferric chloride, U. S. P. lump, was taken up in about 500 cc. ethyl ether and a little water and the solution poured over 1 kg. of wheat germ. The mass was shaken in a closed container, allowed to stand over night and extracted with ether.

*α-Tocopherol.* This material was kindly presented to us by Merck and Co. through the curtesy of Dr. Randolph T. Major.

*Method of inducing muscular dystrophy in rats.* Young female rats were reared on diet I from the time of weaning and bred at 90 days of age according to the technic of Evans and Burr ('27). In order to supply sufficient vitamin E to produce a litter, 1 gm. of wheat germ oil was given by mouth during the first 5 days of gestation. Although the feeding of 550 mg. of wheat germ oil to rats on vitamin E-low diets results in the birth of living young (Evans and Burr, '27), 1 gm. was fed in these experiments in the hope of obtaining more young rats that were viable.

Under these conditions, reproduction compared favorably with that found in normal stock rats. Fifty gestations resulted in forty-seven litters and three resorptions. The litters averaged 5.9 rats, since a total of 275 young were born. Thirty-three (12%) were born dead. The average birth weight of those born alive was 4.8 gm.

All of the young were kept for lactation during which period the diet of the mother was changed to diet II. During the first 10 days of lactation there was a mortality in the young, from unknown causes, of 25%. At the end of the period, from the nineteenth to the twenty-third day, symptoms of paralysis such as described by Evans and Burr ('28) appeared in many of the remaining rats. Among 143 young, 130 (90%) developed the disease and forty-six (35%) of those presenting symptoms died.

On the twenty-fifth day of lactation all of the young were killed. Autopsies were performed on every rat in the litter and representative muscle specimens preserved for histological examination.

*Pathology.* We are indebted to Dr. A. M. Pappenheimer for his study of the pathology of the muscle which will appear in detail elsewhere. The findings confirm those of Olcott ('38) in all essentials. The changes are strictly limited to the voluntary muscles.

*Muscle creatine.* In degenerated muscle of the rat, there is a striking loss of muscle creatine comparable to that found in nutritional muscular dystrophy of the rabbit (Goettsch and

Brown, '32; Morgulis and Spencer, '36) and the guinea pig (Madsen, '36). Creatine was determined by the method of Ross, Helmer and Chanutin ('27). The mean of twenty-eight creatine determinations in normal muscle of 25-day-old rats was 434.2 $\pm$ 4.4 mg. creatine per 100 gm. of fresh muscle; the standard deviation was $\pm$34.2 $\pm$ 3.1 and the coefficient of variation 7.9. Normal muscle of adult rats had the same creatine content. During the first 21 days after birth, the muscle creatine in young rats increased from 140 mg. to the adult level. At 15 days of age, the muscle creatine averaged 300 mg.

In rats with severe muscular dystrophy, the muscle creatine was reduced to as low concentrations as 20 to 30 mg. per 100 gm. fresh tissue (table 1). The decrease corresponded roughly to the duration of the symptoms and to the amount of degeneration in the muscle. During recovery, when the muscle fibers were regenerating, the muscle creatine increased to the normal amount.

*Test for the muscle dystrophy preventing factor.* In testing for the muscle dystrophy preventing factor, some of the young of each litter were kept as controls and were not given any supplementary feeding. The remaining rats were fed the material to be tested. It was given by mouth daily from the tenth to the twenty-fifth day of lactation (Morelle, '31).

Table 1 illustrates not only the variation in extent of muscle lesions among those rats of a litter which had been given no supplementary feeding, but also the prevention of muscular dystrophy in those which had been fed small amounts of active preparations.

A total of forty-seven litters was used for muscle dystrophy preventing tests. Among these, there were forty litters in which all of the control rats presented degenerated muscles; five litters in which some of the controls were affected and some were normal; and only two litters in which none of the control rats developed muscular dystrophy.

TABLE 1

*Showing the extent of degeneration and creatine content of muscles in normal young and those with nutritional muscular dystrophy*

| NUMBER OF LITTER | NUMBER OF YOUNG RAT | AGE IN DAYS | DOSAGE DURING LACTATION | SYMPTOMS MUSCLE DYSTROPHY | | EXTENT OF MUSCLE LESIONS | | MILLIGRAMS CREATINE PER 100 GM. FRESH MUSCLE |
|---|---|---|---|---|---|---|---|---|
| | | | | Extent | Duration | Gross | Microscopic | |
| 21–3 | 1 | 21 | None | ++++ | 2 days | ++++ | ++++ | 70 |
| | 2 | 21 | None | +++ | 2 days | +++ | ++++ | 165 |
| | 3 | 25 | 160 mg. W.G.O. | — | | — | — | 425 |
| | 4 | 25 | 160 mg. W.G.O. | — | | — | — | 430 |
| 36–3 | 1 | 24 | None | ++++ | 1 day | ++ | ++++ | 150 |
| | 2 | 24 | None | ++++ | 1 day | ++ | ++ | 290 |
| | 3 | 25 | 300 mg. T.W.G.O. | ++ | 4 days | ++ | ++++ | 220 |
| | 4 | 25 | 300 mg. T.W.G.O. | — | | + | +++ | 385 |
| 77 | 1 | 25 | None | — | | — | — | 405 |
| | 2 | 25 | None | ++ | 4 days recovered | + | ++ regen. | 300 |
| | 3 | 25 | None | ++ | 4 days recovered | + | +++ regen. | 385 |
| 76 | 1 | 21 | None | ++++ | 2 days | +++ | ++++ | 60 |
| | 2 | 21 | None | ++++ | 2 days | +++ | ++++ | 135 |
| | 3 | 21 | None | ++ | 2 days | ++ | +++ | 380 |
| 35 | 1 | 23 | None | ++++ | 2 days | +++ | +++ | 115 |
| | 2 | 23 | None | ++++ | 4 days | ++++ | +++ | 130 |
| | 3 | 25 | 300 mg. T.W.G.O. | — | | — | regen. | 400 |
| | 4 | 25 | 300 mg. T.W.G.O. | — | | — | — | 455 |
| | 5 | 25 | 300 mg. W.G.O. | — | | — | — | 435 |
| | 6 | 25 | 300 mg. W. G. O. | — | | — | — | 455 |
| 37 | 1 | 19 | None | ++++ | 1 day | + | ++ | 205 |
| | 2 | 21 | None | ++++ | 2 days | ++++ | ++++ | 20–30 |
| | 3 | 24 | 5 mg. α-tocopherol | — | | — | — | 465 |
| 65 | 1 | 25 | None | +++ | 2 days recovered | ++ | +++ regen. | 340 |
| 4 | 1 | 25 | None | +++ | 6 days | ++ | +++ | 295 |
| | 2 | 25 | None | ++ | 5 days recovered | + | regen. | 415 |
| | 3 | 25 | None | — | | — | — | 405 |
| 8–2 | 1 | 25 | None | +++ | 3 days recovered | ++ | ++ regen. | 350 |

Rats that were protected from muscle dystrophy grew as well as rats upon the stock diet. Their average body weight at 21 days was 40 gm.

It was possible to use several litters from the same female rat. After lactation, the rat was given diet I, allowed to rest for about 2 weeks, bred, and given 1 gm. of wheat germ oil. The resulting litter was used for another muscle dystrophy preventing test. In some instances, three litters were used with consistently good results.

*Results of feeding preparations.* Wheat germ oil, oil of treated wheat germ in which vitamin E had been inactivated by ethereal ferris chloride and α-tocopherol were fed under the specified conditions. Preliminary experiments indicated that all three preparations prevented muscular dystrophy in young rats. The final results are given in table 2.

*Wheat germ oil.* Ten rats in four litters were protected from muscle dystrophy when 2 drops of wheat germ oil (a total amount of 600 mg.) were given daily from the tenth to the twenty-fifth day of lactation. When the amount fed was decreased to 1 drop of wheat germ oil daily (a total of 300 mg.), fifteen out of sixteen rats in seven litters were protected. These results are in accord with those of Evans and Burr ('28).

*Oil of treated wheat germ.* The oil extracted from wheat germ that had been treated with ferric chloride also prevented muscle dystrophy, but a larger amount was necessary. Twelve rats in six litters, receiving 2 drops daily (a total of 600 mg.), were protected against the disease. When 1 drop daily (a total of 300 mg.) was given, only six of twelve rats in five litters remained normal.

Since 300 mg. of oil of treated wheat germ was not so active as 300 mg. of untreated wheat germ oil in protecting the rats against degenerated muscle, it is apparent that ferric chloride treatment had inactivated some of the muscle dystrophy preventing factor.

This oil extracted from treated wheat germ was tested for vitamin E potency in the usual manner. It failed to facilitate

the production of living young in twenty rats that had had resorptions on diet I. The following amounts were given: 1 gm. per gestation to seven rats; 2 gm. to three rats; 12 gm. to four rats, one of which had a litter of two dead young; and 20 gm. to six rats. That these rats retained their normal response to vitamin E was proved by the fact that litters

TABLE 2

*Showing the protective effect of wheat germ oil, oil of treated wheat germ and α-tocopherol in nutritional muscular dystrophy in the young rat*

| FEMALE RAT | NO SUPPLEMENT | | WITH SUPPLEMENT | | DOSE |
|---|---|---|---|---|---|
| | Number of rats | Number with muscular dystrophy | Number of rats | Number with muscular dystrophy | |
| 21–2 | 2 | 2 | 3 | 0 | 600 mg. wheat germ oil |
| 36–2 | 2 | 2 | 3 | 0 | 600 mg. wheat germ oil |
| 80–2 | 3 | 3 | 2 | 0 | 600 mg. wheat germ oil |
| 64 | 2 | 1 | 2 | 0 | 600 mg. wheat germ oil |
| 80–2 | 3 | 3 | 2 | 1 | 300 mg. wheat germ oil |
| 80–3 | 2 | 1 | 2 | 0 | 300 mg. wheat germ oil |
| 29–2 | 3 | 3 | 3 | 0 | 300 mg. wheat germ oil |
| 8–2 | 3 | 2 | 2 | 0 | 300 mg. wheat germ oil |
| 35–2 | 2 | 2 | 2 | 0 | 300 mg. wheat germ oil |
| 77 | 3 | 2 | 2 | 0 | 300 mg. wheat germ oil |
| 76 | 3 | 3 | 3 | 0 | 300 mg. wheat germ oil |
| 35–1 | 2 | 2 | 3 | 0 | 600 mg. of oil of treated wheat germ |
| 9 | 2 | 2 | 3 | 0 | 600 mg. of oil of treated wheat germ |
| 12 | 2 | 2 | 3 | 0 | 600 mg. of oil of treated wheat germ |
| 36–2 | 2 | 2 | 3 | 0 | 600 mg. of oil of treated wheat germ |
| 80–3 | 2 | 1 | 3 | 1 | 300 mg. of oil of treated wheat germ |
| 45 | 3 | 3 | 2 | 2 | 300 mg. of oil of treated wheat germ |
| 8–2 | 3 | 2 | 3 | 0 | 300 mg. of oil of treated wheat germ |
| 35–2 | 2 | 2 | 2 | 1 | 300 mg. of oil of treated wheat germ |
| 36–3 | 2 | 2 | 2 | 2 | 300 mg. of oil of treated wheat germ |
| 37–2 | 2 | 2 | 1 | 0 | 5 mg. α-tocopherol |
| 54 | 1 | 1 | 2 | 0 | 5 mg. α-tocopherol |
| 77 | 3 | 2 | 3 | 0 | 5 mg. α-tocopherol |
| 76 | 3 | 3 | 3 | 0 | 5 mg. α-tocopherol |
| 65 | 3 | 1 | 3 | 0 | 5 mg. α-tocopherol |
| 7–3 | 3 | 3 | 3 | 3 | 0.5 mg. α-tocopherol |
| 66–2 | 3 | 3 | 3 | 3 | 0.5 mg. α-tocopherol |
| 29–3 | 2 | 1 | 2 | 1 | 0.5 mg. α-tocopherol |
| 67–2 | 3 | 3 | 3 | 1 | 0.5 mg. α-tocopherol |

were subsequently born to most of them after administration of wheat germ oil during the gestation period.

Since 1 gm. of wheat germ oil permitted normal pregnancies in rats on an E-low diet and since the oil from the treated wheat germ failed, even when fed in amounts as great as 20 gm. per gestation, it is clear that the ferric chloride treatment had inactivated most of the vitamin E. These results are in agreement with those of Waddell and Steenbock ('31), who obtained a marked inactivation of vitamin E in wheat germ by treating the germ with an ether solution of ferric chloride. However, these authors found only a partial destruction of vitamin E activity in samples of wheat germ oil that had been treated with ethereal ferric chloride.

*α-Tocopherol.* The α-tocopherol was dissolved in 47 parts of ethyl laurate and 1 drop of the solution given daily for 15 days to each of twelve rats in five litters. A total amount of 5 mg. of α-tocopherol was given each rat. The controls in these litters were given daily 1 drop of ethyl laurate. All rats receiving the α-tocopherol were protected. The remainder of the solution of α-tocopherol was diluted with 9 parts of ethyl laurate and 1 drop of the resulting solution was fed daily for 15 days (total dose 0.5 mg.) to eleven rats in four litters. Only three rats were protected.

## DISCUSSION

Since muscular dystrophy in rats occurs upon diets that are known to be low in vitamin E, and since protection is afforded by wheat germ oil and α-tocopherol, it would appear that the muscle lesions are due to a deficiency of the antisterility vitamin. However, the results with oil of treated wheat germ, which in adequate dosage prevented degeneration of muscle in young rats but failed to facilitate the birth of living young in adult females, raise certain new problems.

There are several possible explanations for this discrepancy:

1. The requirement of vitamin E for successful reproduction in an adult female may be much greater than that of the young rat for normal muscular development.

2. The young rat may differ in its ability to utilize possible precursors for vitamin E synthesis.

3. Several substances are known to have vitamin E activity (Evans, Emerson and Emerson, '38). There may be several substances that protect against muscular dystrophy, and it is conceivable that the α-tocopherol employed may have contained impurities that are active in preventing the muscle disease.

Inasmuch as the onset of the lesions of nutritional muscular dystrophy in Herbivora and ducklings is prevented or delayed by the inclusion of vegetable oils in the diet (Madsen, '36; Pappenheimer and Goettsch, '36; Mattill, '38), it will be of interest to feed α-tocopherol to these species.

The observation that muscle dystrophy occurs in young rats on synthetic diets low in vitamin E, but not on natural foods diets in which the vitamin E has been destroyed by ethereal ferric chloride, is confirmed by similar experiments upon goats. Thomas and Cannon ('37) fed a treated natural foods diet, on which male rats became sterile in 5 months and females presented typical resorption gestations, to a herd of goats for 4½ years. Normal third generation kids were produced. On the other hand when Madsen, McCay and Maynard ('35) fed kids from the time of weaning on a diet composed of regenerated cellulose, casein, sucrose, starch, yeast, salt mixture and lard, the animals died after 4½ to 10 months. Histological studies showed skeletal muscle lesions in all of the animals.

The contention of Morgulis, Wilder and Eppstein ('38) that two factors, vitamin E and B complex, are necessary for the prevention of muscular dystrophy in rabbits does not apply to the rat disease.

## SUMMARY

A diet is described on which 90% of rats at the end of lactation suddenly become affected with severe degeneration of the skeletal muscles.

A method is described for testing the protective effect of preparations against muscular dystrophy.

Young rats are protected against the muscle disease by supplementing the diet with wheat germ oil, with oil of treated wheat germ in which the anti-sterility vitamin has been inactivated by ethereal ferric chloride or with α-tocopherol.

The activity of a preparation in preventing muscular dystrophy does not necessarily correspond to its anti-sterility potency.

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# ELECTROLYTE AND NITROGEN METABOLISM IN PREGNANCY

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FOUR FIGURES

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The very fact that nature provides a mechanism for temperature control with loss of water and salts through the skin makes electrolyte balance measurements only approximations even when the subjects carry on a sedentary existence. When the subjects are engaged in the activities of daily life, under varying conditions of temperature and humidity, how much more variable must be the losses of these substances! Because of the vagaries of human nature the most careful and continual surveilance is almost an absolute requisite in order to preclude all but the unusual subject from taking liberties that she might regard as inconsequential in effect. The quantitative determination of sodium and potassium is not one of the more simple chemical procedures. And yet, precise as are the analytical methods used in some of the experiments reported in the literature, it is felt that some of the studies have lost much of their potential value because the subjects assumed a large share of the responsibility in the preparation of the diet and collection of excreta.

Coons and co-workers ('34) observed an average daily retention of 1.26 gm. of sodium and 0.51 gm. of potassium in pregnant women who lived in their domestic environments and subsisted on a more or less self chosen diet. Hummel and co-workers ('36) observed an average daily retention of 0.56 gm. of sodium and 1.40 gm. of potassium over a prolonged period during pregnancy. The subject's diet, however, was

self chosen. The data of Freyberg, Reekie and Folsom ('38) show an average daily retention of 0.17 gm. of sodium, allowance being made for loss through the skin. Coons and Hummel reported average daily retentions of 0.89 gm. and 0.60 gm. of chloride respectively. Evidence cited by Wilson ('16) and Stander ('36) indicates that nitrogen is stored in pregnancy, often in excess of the needs of the fetus. Nitrogen balance studies were included in the present investigation to determine any relationship between the storage of this element and that of potassium, sodium and chloride.

## GENERAL METHODS

*Care of patient.* These studies were conducted on two women. Observations on one of them were made during the fourth and eighth months of pregnancy over 10- and 11-day periods respectively. The other subject had mild symptoms of toxemia with moderate edema and elevation of blood pressure. On her the observation period had to be terminated after 5 days because of the onset of labor at term. These subjects were maintained in a special study division under practically constant supervision and observation by nurses trained in metabolic work. On admission a complete physical and laboratory examination was made. Three days were allowed for the subjects to accustom themselves to the diet and other matters of strict routine before actual determinations were begun. The usual bedroom diversions were permitted, but exercise more vigorous than walking slowly was not allowed. At no time was visible perspiration noted even in the axillae.

The study periods began and ended at 8 o'clock A.M. on consecutive days. At 8 o'clock A.M. after voiding, the subjects were weighed on a balance sensitive to 10 gm. Blood samples were then taken as needed and weighed. A given amount of weighed food and distilled water was given at regular intervals, i.e., 8.30 A.M., 1.30 P.M. and 6 P.M., care being taken that the entire diet including the rinsings of the dishes with distilled water from the total alotted quota, and nothing else, was consumed.

*Clinical histories.* Experiment I. H.R., no. 115937. The subject was a 28-year-old primigravida in her fourth month of pregnancy. Her past medical history indicated good average health. Her pregnancy likewise had progressed entirely normally. Examination showed her to be in excellent physical condition. The blood counts and urinary findings were likewise normal. The B.M.R. however was —17%. Metabolic period began on June 22, 1936. Weight 53.21 kg. Diet was well tolerated. Stools were passed every second day and were sharply demarcated with carmine. Study completed on July 2, 1936. Weight 53.19 kg.

*Experiment II.* Same subject as in experiment I, but now in the eighth month of pregnancy. Interval note: The pregnancy had progressed without toxic manifestations or other complications. Examination showed her to be in good health. Metabolic period began on September 25, 1936. Weight 57.16 kg. B.M.R., —18%. Diet well tolerated. Study period of 11 days terminated on October 6, 1936. Weight 57.21.

*Experiment III.* D.V., no. 12516. This subject, a 40-year-old para 1, was admitted 7 days prior to her expected date of confinement. Her past health had been good and there was no history of serious illness. In early pregnancy she had moderately severe nausea and vomiting. Several days before admission her blood pressure rose from 100/60 to 152/88, and for the first time ankle edema was noticed. A mild albuminuria was present. The patient appeared to be a suitable subject typifying pre-eclampsia. Examination showed her to be in good general physical condition. Blood pressure 158/85. Moderate pre-tibial edema present. Blood counts normal. Blood N.P.N. 26 mg.%. The urine contained about ½ gm. of albumin per 24-hour specimen, but was otherwise normal. B.M.R. +12%. Metabolic period began October 9, 1936. Weight 73.54. Diet well taken, but subject had numerous diarrhoeal stools. Complete data over a 5-day period were available, but the study had to be subsequently terminated because of the onset of labor. Weight at close of period 72.97 kg.

*Diet.* The standard daily diet in each of the three experiments was as follows:

| | |
|---|---|
| Distilled water | 2500 gm. |
| Powdered whole milk | 252 gm. |
| Lactose | 36 gm. |
| Chocolate malted milk | 20 gm. |
| Graham crackers | 50 gm. |
| Tomato juice | 400 gm. |
| Dextrin | 20 gm. |
| Sucrose | 10 gm. |
| Sodium chloride, 10% sol. | 30 cc. |

These foods were of standard brands and were pooled from containers having the same lot numbers. They were prepared in sufficient quantities to insure against the need of replenishment from outside sources during the course of the experiment and stored in the refrigerator. The absolute uniformity obviated the need of repeated analyses. The composition of the stools was consequently likewise quite uniform. This diet contained protein approximately 70 gm., fat 77 gm., carbohydrates 230 gm., calcium 2.17 gm., phosphorus 1.84 gm., iron 0.005 gm., and was complete as regards vitamins A, B, C and G. All items of the diet, in each experiment were analysed for moisture, potassium, sodium, chlorides and nitrogen.

*Collection of blood.* Blood was withdrawn by vena-puncture without stasis and collected under oil after a 12-hour fast at the beginning and conclusion of a metabolic period. The serum was analysed for Na, K, Cl, N and total base.

*Collection of excreta.* Urine. The urine was passed directly into a bottle packed in ice and containing 10 cc. of glacial acetic acid. Aliquots from the mixed 24-hour specimen were analysed for Na, K, Cl and N. Stools. The stools were sharply demarcated with carmine and stored in a vessel containing 10 cc. of glacial acetic acid and a measured amount of distilled water. All stools not relating to the experiment were discarded. Because of the irregularity of movement of the bowls, the total fecal constituents passed over the entire metabolic period were pro-rated on a daily basis. The stool-

water mixture was subjected to prolonged mechanical agitation and analysed for Na, K, Cl and N.

*Analytical methods.* Sodium was determined by the method of Barber and Kolthoff ('28) as modified by Salit ('32), and potassium by the method of Shohl and Bennett ('28) as modified by Hald ('33). Chloride in the food was determined by the method of Birner ('28); and in the urine and feces by a modified Volhard-Harvey titration. Total base was determined by the method of Keys ('36). Nitrogen was determined by the Kjeldahl procedure.

## RESULTS AND DISCUSSION

The data are presented in table 1. An advantage of the diet used is indicated by the constant daily input of electrolyte and nitrogen. Variations in the output of these elements must therefore have another basis. With this diet, the subjects maintained their approximate weights. A small amount of weight loss frequently occurs just prior to the onset of labor at term. The weight loss in experiment III may have been caused by excessive water loss due to diarrhoea.

The data indicate a discernable retention of sodium. The average daily retentions of sodium were found to be 19.3 m. eq. in the fourth month and 11.6 m. eq. and 13.2 m. eq. in the eighth and ninth months, respectively. The average daily retention values for potassium were 8.4, 10.4 and 5 m. eq. in the same periods. No constant upward or downward trend is noted in the curve for chloride accumulation, the average daily balances of chloride being −5.5, +1.2 and +4 m. eq., respectively. Apparently some other anion than chloride must be linked with the stored potassium.

It is noted that the sum of the sodium and potassium in the serum approximates the values for total base. But when one considers from the calibration of the methods that sodium recovery was 2.7% high and total base recovery was 1.3% low, the apparent discrepancy between sodium, potassium and total base is largely removed. Appreciable changes in serum electrolytes were not observed.

TABLE 1

*Daily electrolyte and nitrogen balances*

| EXPERIMENT | DATE | WT. | REL. HUM. | TEMPERATURE °F. | INPUT | | | | OUTPUT | | | | BALANCE | | | | SERUM PER LITER | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Na | K | Cl | N | Na | K | Cl | N | Na | K | Cl | N | Na | K | Cl | Total base | N |
| | | *kg.* | % | | *m. eq.* | *m. eq.* | | *gm.* | *m. eq.* | *m. eq.* | | *gm.* | *m. eq.* | *m. eq.* | | *gm.* | *m. eq.* | *m. eq.* | | | *gm.%* |
| | 6/22/36 | 53.21 | 48 | 72 | 158 | 105 | 164 | 11.23 | 141 | 98 | 201 | 12.48 | 17 | 7 | —37 | —1.25 | 141.0 | 4.7 | 104.6 | 145.1 | 1.07 |
| | 6/23/36 | 53.27 | 51 | 72 | 158 | 105 | 164 | 11.23 | 141 | 98 | 178 | 12.20 | 17 | 7 | —14 | —0.97 | | | | | |
| | 6/24/36 | 53.03 | 55 | 71 | 158 | 105 | 164 | 11.23 | 141 | 98 | 168 | 12.35 | 17 | 7 | —4 | —1.12 | | | | | |
| No. 1 | 6/25/36 | 53.29 | 57 | 73 | 158 | 105 | 164 | 11.23 | 139 | 97 | 129 | 11.83 | 19 | 8 | 35 | —0.60 | | | | | |
| H.R. | 6/26/36 | 53.34 | 67 | 76 | 158 | 105 | 164 | 11.23 | 117 | 96 | 147 | 12.29 | 41 | 9 | 17 | —1.06 | | | | | |
| 4 months | 6/27/36 | 53.45 | 72 | 77 | 158 | 105 | 164 | 11.23 | 124 | 97 | 160 | 11.02 | 34 | 8 | 4 | 0.21 | | | | | |
| preg- | 6/28/36 | 53.38 | 56 | 74 | 158 | 105 | 164 | 11.23 | 157 | 101 | 182 | 11.36 | 1 | 4 | —18 | —0.13 | | | | | |
| nancy | 6/29/36 | 53.16 | 57 | 72 | 158 | 105 | 164 | 11.23 | 150 | 89 | 186 | 10.94 | 8 | 16 | —22 | 0.29 | | | | | |
| | 6/30/36 | 53.38 | 53 | 73 | 158 | 105 | 164 | 11.23 | 127 | 85 | 156 | 11.31 | 31 | 20 | 8 | —0.08 | | | | | |
| | 7/1/36 | 53.19 | 53 | 72 | 158 | 105 | 164 | 11.23 | 150 | 107 | 188 | 11.48 | 8 | —2 | —24 | —0.25 | | | | | |
| | 7/2/36 | | | | | | | | | | | | | | | | 142.4 | .. | 102.1 | 144.7 | 1.03 |
| | | | | | | | | | | Total retention | | | 193 | 84 | —55 | —4.96 | | | | | |
| | | | | | | | | | | Av. daily ret. | | | 19.3 | 8.4 | —5.5 | —0.50 | | | | | |
| | 9/25/36 | 57.16 | 51 | 75 | 145 | 107 | 166 | 11.42 | 134 | 92 | 174 | 11.58 | 11 | 15 | —8 | —0.16 | 139.8 | 5.5 | 106.3 | 145.0 | 0.96 |
| | 9/26/36 | 57.20 | 51 | 77 | 145 | 107 | 166 | 11.42 | 135 | 104 | 177 | 11.40 | 10 | 3 | —11 | 0.02 | | | | | |
| | 9/27/36 | 57.41 | 65 | 77 | 145 | 107 | 166 | 11.42 | 119 | 103 | 156 | 10.96 | 26 | 4 | 10 | 0.46 | | | | | |
| | 9/28/36 | 57.33 | 53 | 77 | 145 | 107 | 166 | 11.42 | 120 | 95 | 163 | 11.02 | 25 | 12 | 3 | 0.40 | | | | | |
| No. 2 | 9/29/36 | 57.28 | 48 | 78 | 145 | 107 | 166 | 11.42 | 137 | 98 | 170 | 10.79 | 8 | 9 | —4 | 0.63 | | | | | |
| H.R. | 9/30/36 | 57.34 | 49 | 78 | 145 | 107 | 166 | 11.42 | 116 | 95 | 147 | 11.69 | 29 | 12 | 19 | —0.27 | | | | | |
| 8 months | 10/1/36 | 57.04 | 56 | 79 | 145 | 107 | 166 | 11.42 | 165 | 101 | 181 | 12.08 | —20 | 6 | —15 | —0.66 | | | | | |
| preg- | 10/2/36 | 57.06 | 53 | 79 | 145 | 107 | 166 | 11.42 | 144 | 89 | 166 | 11.31 | 1 | 18 | 0 | 0.11 | | | | | |
| nancy | 10/3/36 | 57.16 | 51 | 78 | 145 | 107 | 166 | 11.42 | 156 | 93 | 175 | 11.38 | —11 | 14 | —9 | 0.04 | | | | | |
| | 10/4/36 | 57.09 | 46 | 78 | 145 | 107 | 166 | 11.42 | 122 | 93 | 150 | 10.55 | 23 | 14 | 16 | 0.87 | | | | | |
| | 10/5/36 | 57.21 | 55 | 80 | 145 | 107 | 166 | 11.42 | 119 | 100 | 154 | 10.28 | 26 | 7 | 12 | 1.14 | | | | | |
| | 10/6/36 | | | | | | | | | | | | | | | | 141.2 | 5.4 | 106.3 | 145.3 | 1.04 |
| | | | | | | | | | | Total retention | | | 128 | 114 | 13 | 2.56 | | | | | |
| | | | | | | | | | | Av. daily ret. | | | 11.6 | 10.4 | 1.2 | 0.23 | | | | | |
| | 10/9/36 | 73.54 | 60 | 75 | 146 | 102 | 168 | 11.53 | 126 | 92 | 160 | 14.21 | 20 | 10 | 8 | —2.68 | 142.4 | 5.4 | 107.0 | 146.6 | 0.96 |
| No. 3 | 10/10/36 | 73.39 | 66 | 74 | 146 | 102 | 168 | 11.53 | 122 | 96 | 156 | 15.08 | 24 | 6 | 12 | —3.55 | | | | | |
| D.V. | 10/11/36 | 73.21 | 56 | 73 | 146 | 102 | 168 | 11.53 | 123 | 100 | 147 | 13.40 | 23 | 2 | 21 | —1.87 | | | | | |
| term | 10/12/36 | 73.40 | 47 | 74 | 146 | 102 | 168 | 11.53 | 146 | 103 | 180 | 12.35 | 0 | —1 | —12 | —0.82 | | | | | |
| preg- | 10/13/36 | 72.97 | 50 | 74 | 146 | 102 | 168 | 11.53 | 147 | 94 | 177 | 12.82 | —1 | 8 | —9 | —1.29 | | | | | |
| nancy | 10/14/36 | | | | | | | | | | | | | | | | 140.8 | 5.6 | 105.6 | 145.8 | 0.93 |
| | | | | | | | | | | Total retention | | | 66 | 25 | 20 | —10.21 | | | | | |
| | | | | | | | | | | Av. daily ret. | | | 13.2 | 5 | 4 | —2.04 | | | | | |

The positive balance of electrolytes illustrated in figure 3 is contrary to what might be expected of a diarrhoeal patient losing ½ kg. in weight. The subject in this experiment was edematous, but it is difficult accurately to measure weight loss due to reduction of edema. Can it be that the demands for electrolytes during pregnancy are strong enought to withstand the consequence of a mild diarrhoea? Although our data do not support the generally accepted evidence of nitrogen storage, it is not to be regarded as a refutation of that evidence. Results must be interpreted as they relate to the conditions of an experiment. Sherman ('26) has emphasized that the body tends to adjust its protein metabolism to its protein supply, and that an appreciable length of time may be required to effect adjustment from a higher to a lower level. Perhaps in these experiments, the relative nitrogen deficit resulted from adjustments to lower protein intake than that to which the subjects had been accustomed. The passage of unassimilated nitrogen in the stool, in experiment III, may have contributed in large measure to the nitrogen deficit. In any event, the subject lost an average of 2.83 gm. of nitrogen daily in the stools, while the subject in experiments I and II lost only 0.95 gm. of N daily by this route.

Daily variations in the balances of electrolytes were noted in all three experiments and are illustrated in figure 4. The sodium and chloride balances in experiment II are seen to parallel one another quite closely; in fact, for 5 days the retentions of these elements were equivalent. This striking parallelism was likewise noted in experiments I and III. Potassium balances varied less than either those of sodium or chloride. There appeared to be no correlation between the potassium and the sodium variation. The variations were probably referable to such unmeasured factors as changes in exercise, humidity and temperature. Freyburg and Grant ('37), using as subjects "men who went about their usual laboratory duties with special precautions only to keep themselves cool enough to prevent sweating" found the daily loss through the skin to be sodium 71 to 209 mg., potassium 133

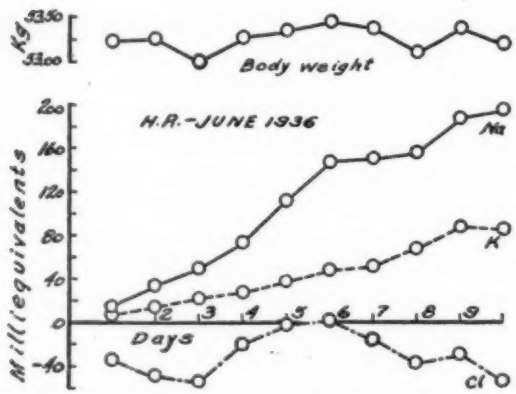


Fig. 1 Showing the cumulative balances in experiment I. Subject H.R., 4 months pregnant.

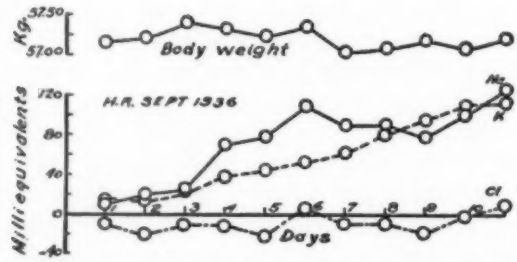


Fig. 2 Showing the cumulative balances in experiment II. Subject H.R., 8 months pregnant.

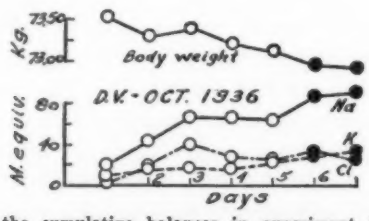


Fig. 3 Showing the cumulative balances in experiment III. Subject D.V., at term. The data for the last 2 days are incomplete because of the sudden onset of labor on the seventh day.

to 179 mg. and chloride 146 to 248 mg. Basset and Keutmann (unpublished data) studied arthritic subjects and found daily losses through the skin to be sodium 180 mg., potassium 239 mg. and chloride 194 mg. daily.

Considerable variation in output of these electrolytes through the skin is thus noted, and while it would be jeopardous to transpose data from one type of experiment to another, it shows the approximate magnitude of electrolyte loss with which we might be expected to contend. It is felt that even when allowance is made for losses through the skin, the evidence presented in this paper suggests definite storage of sodium. The evidence for potassium and chloride storage is less conclusive.

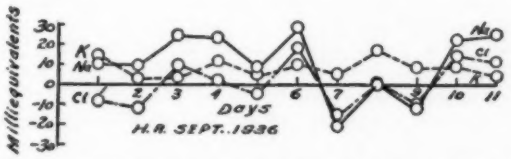


Fig. 4 Showing diurnal variations in retention of electrolytes in experiment 2. Experiments I and III likewise show comparable variations.

## SUMMARY

Electrolyte and nitrogen balance studies were conducted under practically constant supervision on two women in the fourth, eighth and ninth months of gestation. The same diet was used in each experiment and was of constant composition from day to day. Diregarding losses through the skin, the average daily retentions of sodium and potassium based on the three metabolic studies were observed to be 14.7 milliequivalents and 7.9 milliequivalents respectively. Fluctuations in potassium retention varied less from day to day than did those of sodium and chloride. Variations in sodium and chloride retention appear to parallel one another. Such a parallelism does not seem to apply to potassium. The subjects were maintained in approximate chloride equilibrium. For only one experiment was a positive nitrogen balance ob-

served. The nitrogen deficit in one of the experiments may be explained on the basis of diarrhea. The need of prolonged periods of continuous observation is stressed.

The authors are happy to acknowledge with gratitude the assistance given in this study by Miss Clara Miller, R.N. and Mrs. Verna Eisenhauer, R.N. Dr. Estelle Hawley has our appreciation for the planning of the diet.

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# A QUANTITATIVE STUDY BY MEANS OF SPECTROGRAPHIC ANALYSIS OF ALUMINUM IN NUTRITION [1]

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ONE FIGURE

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Whether aluminum is present in plant and animal matter formed the basis of a controversy between McCollum, Rask and Becker ('28) and Kahlenberg and Closs ('29). Both used the spectrographic technic in their studies. The former used only the spark to obtain the emission of light from the elements, while the latter used both the arc and spark methods of exciting the elements. McCollum, Rask and Becker ('28) concluded from the inspection of their spectrograms that aluminum is a constituent neither of plant nor animal matter. Kahlenberg and Closs ('29) on the other hand reported the presence of aluminum in these materials when both the spark and arc were employed. Whether the length of the arcing or sparking period was an influencing factor in determining the presence of aluminum in these materials is not known from the published data. Recent work of Harrison ('36) indicates that the different elements require different lengths of excitation time before emitting light. He found that the aluminum lines were much more intensified after 80 seconds of excitation. The findings of Kahlenberg and Closs, however,

[1] The data of this study are taken from a dissertation submitted in partial fulfillment of the requirements for the degree of doctor of philosophy in nutrition, Child Welfare Research Station, State University of Iowa.

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have been confirmed by Underhill and Peterman ('29) and by Cox and co-workers ('32). The earlier literature which has been summarized by Smith ('28) indicates that aluminum is widespread in nature.

According to Lewis ('31) human blood is free from aluminum or contains only 1 part in five or ten millions, while Underhill and Peterman ('29) reported that aluminum is always present in blood of normal man, the average concentration being 0.060 mg. per 100 ml. of blood. Most investigators agree that there is no convincing evidence that small amounts of aluminum are harmful to the normal individual, but that large amounts may be. However, Myers and Mull ('28) found that when 8 mg. of aluminum per kilogram of body weight, two to three times the amount the Referee Board ('14) considered a large amount, was given rats the minute traces of aluminum normally present in tissues was only slightly increased. Furthermore, Tocco and Mulas ('35) concluded from their work that aluminum powders produced by mechanical cleaning of cooking utensils did not have injurious effects when ingested or injected into frogs and rabbits.

All evidence to date is to the effect that aluminum in the amounts present in foods, either normally or after cooking is harmless. There is little evidence to show whether or not it is an essential constituent of the daily diet. It seemed possible that this might be determined through balance studies with children.

The purpose of the present study was to determine the amount, if any, of aluminum retained by children of preschool age. The spectrograms of the ashed food, feces, urine, distilled water and acid alcohol were compared with spectra produced by solutions of known aluminum content.

## MATERIALS AND METHODS

The diets employed, the care used in handling and collecting of the food and excreta, the preparation of aliquots for spectrographic analysis and the method used in reading the

spectrograms is given by Scoular ('38). A portion of that description is repeated here.

During the entire study only two major changes were made in the original diet (diet I) which consisted of a whole grain bread and cereal, butter, green beans, carrots, potatoes, bananas, apple sauce, prunes, eggs, ground beef and milk. Each child also received specified amounts of Viosterol, cod liver oil and orange juice daily. The first change (diet II) consisted in substituting white bread and Cream of Wheat for the whole grain bread and cereal, tomatoes for green beans, and decreasing the amount of orange juice by one-half. The second change (diet III) consisted in a reduction in the amount of meat and egg and an increase in the quantity of fruit and vegetables and butter. Whenever possible, the necessary adjustments for the increased caloric needs of the child were made by slight changes in sugar.

The duplicate 4 gm. samples of dried food first used were increased to 6 gm. when it was found that the smaller samples gave spectrographic lines for copper which were less distinct and more difficult to read. Two grams of the dried feces and 500 ml. of urine were used throughout the study. Both the food and the feces were ashed for 12 hours at dull red heat in a muffle furnace, whereas the urine was ashed in the air with the aid of two Fischer burners. Five hundred milliliter samples from each carboy used of distilled water, as well as acid-alcohol were evaporated and ignited prior to dilution.

Each of the ashed samples was dissolved in 3 ml. of hydrochloric acid and made up to a volume of 10 ml. in calibrated Pyrex volumetric flasks with redistilled water.

A known weight of aluminum which had been precipitated by 8-hydroxy-quinoline [3] was dissolved in hydrochloric acid and made up to a given volume with redistilled water. The difference between the length of the aluminum line, 3961.5 Angstroms, and the reference bands, 3889 Angstroms, was read from spectrograms which were made from graded con-

[3] Method taken from Organic Reagents for Metals, Staff Research Laboratory of Hopkins and Williams, Ltd., 1933.

centrations of this solution. These differences were plotted for a given concentration and used in determining the amount of aluminum in the unknowns.

The effect of the sodium chloride, present in the unknown solutions, upon the length of both the copper and zinc lines (Scoular, '38, '39) suggested that it might have an effect upon the aluminum line. The influence of sodium chloride, therefore, was tested. Two sets of electrodes were prepared from the special spectrographic quality graphite, to each of which was added the same amount of aluminum. To one of these there were added in addition to the aluminum 4 drops of a saturated solution of sodium chloride. When the spectrograms of these two sets of electrodes were compared, it was found that the sodium chloride produced less difference between the aluminum line 3961.5 Angstroms and the reference bands at 3889 Angstroms, than was obtained from the spectra of aluminum alone. This effect was similar to that experienced with copper and zinc when sodium chloride was arced with these. Therefore, both aluminum lines were plotted for a given concentration (fig. 1). When 4 drops of sodium chloride were used it was possible to read a concentration of 0.00005 mg. of aluminum per milliliter, while without the addition of the sodium chloride to the electrode, 0.0005 mg. was the lowest concentration at which this wave length could be read. The effect of the sodium chloride therefore, was to lessen the distance between the aluminum line 3961.5 Angstroms and the reference bands at 3889 Angstroms.

Since the wedge spectrograms used in the copper study were also used to obtain the aluminum retentions, all precautions which were employed to prevent contamination with copper, also prevented the addition of extraneous aluminum.

The most sensitive aluminum line 3961.5 Angstroms was used to read both the standards and the unknowns since it was the only aluminum line appearing on all the wedge spectrograms. Because of the previously mentioned effect of sodium chloride upon the difference between the aluminum line and the reference bands, the concentration of the aluminum in the

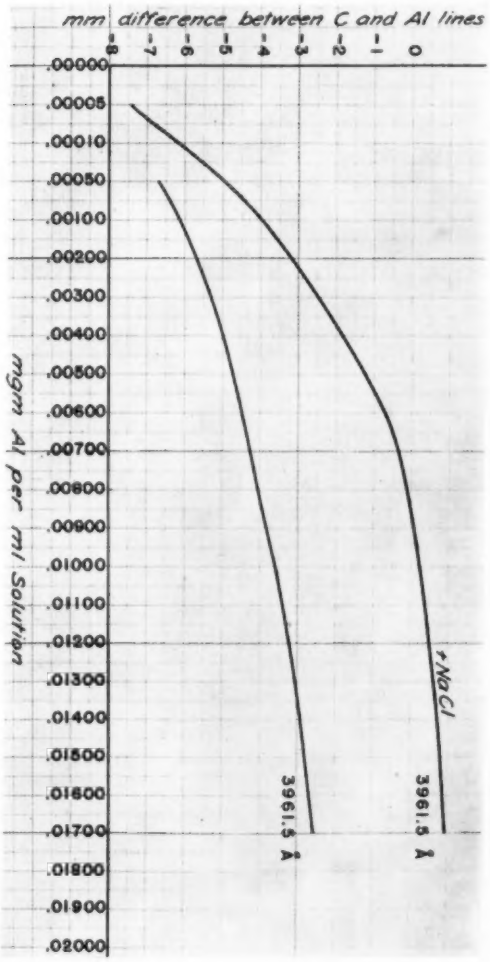


Fig. 1 Aluminum concentration, in milligrams plotted for each tenth of a millimeter difference between the length of the zinc line and the reference bands, in spectra produced by standard solutions, with and without the addition of sodium chloride.

unknowns was determined as for copper (Scoular, '38) by reading the 'best-fit' curves.

The spectrograms of the distilled water used for drinking purposes showed the water to contain a variable amount of aluminum, namely 0.001 to 0.015 mg. per 500 milliliters. The cause of the wide range of aluminum content of the distilled water may be explained by the length of time the water stood in the carboys, or by the fact that from time to time new carboys may have been used. The acid-alcohol used on the feces and food, however, contributed from 0.001 to 0.003 mg. per 100 ml. Since in all cases the water used was tested and measured, these findings did not invalidate the results.

The 'intensity' of the food spectra was very indistinct; this combined with the incandescent effect of the graphite electrode on the spectrogram made it difficult to read the end of the line. Lamb ('35) also observed this effect. Three or more readings of each line were necessary. The average of these was used in comparing the differences between the aluminum line and the carbon bands of the duplicate spectrograms made of each aliquot.

To test the method employed in this study, 0.005 mg. of aluminum was added to duplicate electrodes of food, feces and urine. Recoveries of from 100 to 110%, 90 to 106% and 90 to 96%, respectively, were obtained (table 1). These findings are much closer than those of Dee and Rask ('31) who reported $\pm 25\%$ error for the spark method. This wide range with the spark method, Lampitt and Sylvester ('32) attribute to the variability of the spark. In the above report of Dee and Rask, the authors suggest tests with the arc as being especially adapted to the analysis of biological materials. However, the $\pm 10\%$ error of recovery reported for the present study represents the recovery of 0.005 mg. of aluminum from samples containing 0.00065 to 0.0057 mg. of aluminum. Recoveries reported by Eveleth and Myers ('36) for the colorimetric methods, aurin and alizarin, were 80 to 95% for alizarin and 80 to 120% for aurin. These represented in all but one instance, the recovery of larger amounts of aluminum by the

aurin method from samples containing from 0.0046 to 0.013 mg. of aluminum. This amount represents from 0 to 4 times as much as was used in the present study. Although the recoveries obtained with the alizarin method were made with the addition of much smaller amounts of aluminum, 0.0005 to 0.002 mg., the analysis of dog tissue by these two colorimetric methods showed that the alizarin-lake gave much higher results than did the aurin-lake. However, Eveleth and Myers ('36) conclude that if all of the interfering substances are removed, comparable results are obtained with these two

TABLE 1

*Amount of added aluminum recovered from samples of food, feces and urine*

| PERIODS | SAMPLES TESTED CONCENTRATION | SAMPLE PLUS 0.005 MG. Al | | RECOVERY |
|---|---|---|---|---|
| | | Concentration | | |
| | *mg.* | *mg.* | *mg.* | % |
| Urine | | | | |
| J.E. 2/9 | 0.0015 | 0.0060 | 0.0045 | 90 |
| G.E. 3/22 | 0.0039 | 0.0085 | 0.0046 | 92 |
| F.V. 3/17 | 0.0032 | 0.0080 | 0.0048 | 96 |
| Feces | | | | |
| J.E. 3/17 | 0.0057 | 0.0110 | 0.0053 | 106 |
| G.E. 3/18 | 0.0012 | 0.0057 | 0.0045 | 90 |
| F.V. 3/22 | 0.0021 | 0.0068 | 0.0047 | 94 |
| Food | | | | |
| J.E. 6/2 | 0.0007 | 0.0057 | 0.0050 | 100 |
| G.E. 4/21 | 0.0010 | 0.0065 | 0.0055 | 110 |
| F.V. 4/21 | 0.0010 | 0.0060 | 0.0050 | 100 |

methods. The recovery results obtained with the spectrographic technic reported in this study indicates that it is more sensitive than the colorimetric methods commonly used in analyzing biological materials for aluminum.

## RESULTS AND DISCUSSION

No attempt was made to vary the ingestion level of aluminum. This was found, however, to be from 0.014 to 0.030 mg. of aluminum per kilogram of body weight. The variation in the aluminum content of the diets varied less with the diet than with the difference in food supply. The effect of the

use of different cooking vessels upon the aluminum content of certain foods was shown by Beal and co-workers ('32). In all instances foods cooked in aluminum utensils had a higher aluminum content than when the same food was cooked in glass. Since the same utensils, bright aluminum, were used for the preparation of all foods used in this study, the amount of aluminum varied only with the food source and its reaction with the metal. This is well illustrated by diet III (table 2) for periods 6/2 and 6/7 when the prepared food furnished from 0.016 to 0.019 mg. of aluminum per kilogram of body weight, while the same diet during periods 4/21 and 4/26 and 5/12 and 5/17 furnished from 0.023 to 0.030 mg. per kilogram. Since the only known difference in these food supplies was the use of 'new' potatoes during the 6/2 and 6/7 periods, it suggests that these contained less aluminum than the old potatoes previously used. Some difference is also shown in the aluminum content of diet II during two consecutive double metabolism periods, but the difference is less marked than with diet III, falling within the range of experimental error for the method employed. The theoretical values of all diets could not be obtained since the published tables of Underhill and Peterman ('29), Winter and Bird ('29), Beal and co-workers ('32) and Cox et al. ('32) were incomplete. Using the available data, diet III for F.V. 4/21 and 4/26 was estimated to contain 12.5 mg. of aluminum, 11.3 mg. of which was estimated as supplied by milk alone. These estimated values were very much higher than the values obtained by spectrographic analysis of the foods used in the diet.

In all instances the urine contained measurable amounts of aluminum, from 0.012 to 0.160 mg. per day. This did not increase with increased ingestion levels. There was a tendency, however, for the urine to show slight changes in aluminum concentration as the aluminum content of the diet changes from one 5-day period to the next successive 5-day period. A range of from 3 to 29% of the ingested aluminum in the present study was excreted in the urine. Flynn and

Inouye ('28) report that 30% of the ingested aluminum was excreted in the urine. These results suggest that aluminum is absorbed in part at least, and excreted by the kidneys.

The ingested aluminum is largely excreted through the alimentary tract, from 38 to 127% in the case of the children studied. Flynn and Inouye ('28) reported an average of 70% excreted in the feces, whereas MacKenzie ('30 and '31) recovered from 74.9 to 114% from the feces of rats and concluded that aluminum was quantitatively excreted through the alimentary tract of rats. During certain periods of the present study more aluminum was excreted than ingested. The source of this excessive fecal aluminum is not known since every precaution was taken to prevent contamination. All but one of the seven balances exhibiting aluminum elimination in excess of that ingested, occurred during the first 5-day metabolism period. There are, however, three instances when the fecal aluminum of the second 5-day period greatly exceeded that of the first period, yet fell well within the amount ingested. These particular cases seem to point to delayed aluminum elimination.

Fifty per cent of the balances are negative, ranging from 0.0003 to 0.0070 mg. per kilogram, with the positive retentions varying from 0.0003 to 0.0053 mg. per kilogram. Sixty per cent of all of these fell within the $\pm 10\%$ error as determined for the method used in the study. The remaining 40% of the aluminum balances do not suggest that aluminum is essential in the diets of preschool age children, since only three of the eight cases are positive. And, since there is no relation between the ingestion level and these slight positive balances, it would seem that aluminum is not essential in the human dietary.

## SUMMARY

Thirty-five aluminum balance studies have been made with three normal boys of preschool age.

The ashed food, feces, urine, distilled water and acid-alcohol were analyzed spectrographically for aluminum by comparing

TABLE 2

*Average daily intake, excretion and retention of aluminum*

| DIET NUMBER | CASE DATE | ALUMINUM INGESTION | | | | | ALUMINUM EXCRETION | | | RETENTION | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Food | $H_2O$ | A. A. | Total | | Feces | Urine | Total | Total | Per kilogram |
| | | | | | *mg.* | Per kilogram | | | | | |
| | | *mg.* | *mg.* | *mg.* | | | *mg.* | *mg.* | *mg.* | *mg.* | *mg.* |
| II | F.V. | | | | | | | | | | |
| | 3/19 | 0.27 | 0.01 | 0.00 | 0.28 | | 0.37 | 0.08 | 0.45 | —0.17 | |
| | 3/22 | 0.29 | 0.01 | 0.00 | 0.30 | | 0.17 | 0.09 | 0.26 | 0.04 | |
| | Average | | | | 0.29 | 0.014 | | | | —0.07 | —0.004 |
| II | J.E. | | | | | | | | | | |
| | 3/17 | 0.27 | 0.01 | 0.00 | 0.28 | | 0.48 | 0.09 | 0.57 | —0.29 | |
| | 3/22 | 0.30 | 0.01 | 0.00 | 0.31 | | 0.18 | 0.10 | 0.28 | 0.03 | |
| | Average | | | | 0.29 | 0.015 | | | | —0.13 | —0.007 |
| II | G.E. | | | | | | | | | | |
| | 3/18 | 0.24 | 0.01 | 0.00 | 0.25 | | 0.12 | 0.09 | 0.21 | 0.04 | |
| | 3/22 | 0.26 | 0.01 | 0.00 | 0.27 | | 0.10 | 0.09 | 0.19 | 0.08 | |
| | Average | | | | 0.26 | 0.015 | | | | 0.06 | 0.003 |
| III | G.E. | | | | | | | | | | |
| | 6/2 | 0.30 | 0.01 | 0.01 | 0.30 | | 0.39 | 0.10 | 0.49 | —0.19 | |
| | 6/7 | 0.24 | 0.01 | 0.01 | 0.24 | | 0.11 | 0.06 | 0.17 | 0.07 | |
| | Average | | | | 0.27 | 0.016 | | | | —0.06 | —0.003 |
| III | F.V. | | | | | | | | | | |
| | 6/2 | 0.39 | 0.01 | 0.01 | 0.39 | | 0.25 | 0.12 | 0.37 | 0.02 | |
| | 6/7 | 0.33 | 0.11 | 0.01 | 0.33 | | 0.30 | 0.10 | 0.40 | —0.07 | |
| | Average | | | | 0.36 | 0.017 | | | | —0.02 | —0.001 |
| III | J.E. | | | | | | | | | | |
| | 6/2 | 0.40 | 0.01 | 0.01 | 0.40 | | 0.49 | 0.10 | 0.59 | —0.19 | |
| | 6/7 | 0.34 | 0.01 | 0.01 | 0.34 | | 0.11 | 0.08 | 0.19 | 0.15 | |
| | Average | | | | 0.36 | 0.018 | | | | —0.02 | —0.001 |
| II | J.E. | | | | | | | | | | |
| | 3/1 | 0.38 | 0.00 | 0.00 | 0.38 | 0.019 | 0.27 | 0.09 | 0.36 | 0.02 | 0.001 |
| II | F.V. | | | | | | | | | | |
| | 2/25 | 0.40 | 0.00 | 0.00 | 0.40 | | 0.62 | 0.07 | 0.69 | —0.29 | |
| | 3/1 | 0.38 | 0.00 | 0.00 | 0.38 | | 0.12 | 0.08 | 0.20 | 0.18 | |
| | Average | | | | 0.39 | 0.020 | | | | —0.06 | —0.003 |
| II | G.E. | | | | | | | | | | |
| | 2/25 | 0.38 | 0.00 | 0.00 | 0.38 | | 0.32 | 0.07 | 0.39 | 0.01 | |
| | 3/1 | 0.33 | 0.00 | 0.00 | 0.33 | | 0.21 | 0.06 | 0.27 | 0.06 | |
| | Average | | | | 0.35 | 0.021 | | | | 0.03 | 0.001 |
| III | G.E. | | | | | | | | | | |
| | 5/12 | 0.41 | 0.02 | 0.01 | 0.42 | | 0.34 | 0.11 | 0.45 | —0.03 | |
| | 5/17 | 0.40 | 0.02 | 0.01 | 0.41 | | 0.28 | 0.09 | 0.37 | 0.04 | |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Average | | | | 0.35 | 0.021 | | | | 0.03 | 0.001 |
| III | G.E. | | | | | | | | | | |
| | 5/12 | 0.41 | 0.02 | 0.01 | 0.42 | | 0.34 | 0.11 | 0.45 | —0.03 | |
| | 5/17 | 0.40 | 0.02 | 0.01 | 0.41 | | 0.28 | 0.09 | 0.37 | 0.04 | |
| | Average | | | | 0.41 | 0.023 | | | | 0.01 | 0.000 |
| I | G.E. | | | | | | | | | | |
| | 2/4 | 0.36 | 0.00 | 0.01[2] | 0.35 | | 0.17 | 0.10 | 0.27 | 0.08 | |
| | 2/9 | 0.41 | 0.00 | 0.01[2] | 0.40 | | 0.34 | 0.09 | 0.43 | —0.03 | |
| | Average | | | | 0.38 | 0.023 | | | | 0.03 | 0.002 |
| I | J.E. | | | | | | | | | | |
| | 2/4 | 0.48 | 0.00 | 0.01[2] | 0.47 | | 0.44 | 0.02 | 0.46 | 0.01 | |
| | 2/9 | 0.51 | 0.00 | 0.01[2] | 0.50 | | 0.47 | 0.03 | 0.50 | 0.00 | |
| | Average | | | | 0.49 | 0.024 | | | | 0.01 | 0.000 |
| I | F.V. | | | | | | | | | | |
| | 2/4 | 0.51 | 0.00 | 0.01[2] | 0.50 | | 0.29 | 0.01 | 0.30 | 0.20 | |
| | 2/9 | 0.54 | 0.00 | 0.01[2] | 0.53 | | 0.52 | 0.10 | 0.62 | —0.09 | |
| | Average | | | | 0.51 | 0.028 | | | | 0.06 | 0.003 |
| III | F.V. | | | | | | | | | | |
| | 5/12 | 0.65 | 0.00 | 0.01 | 0.64 | | 0.81 | 0.10 | 0.91 | —0.27 | |
| | 5/17 | 0.55 | 0.00 | 0.01 | 0.54 | | 0.36 | 0.16 | 0.52 | 0.02 | |
| | Average | | | | 0.59 | 0.029 | | | | —0.12 | —0.006 |
| III | F.V.[1] | | | | | | | | | | |
| | 4/21 | 0.54 | 0.00 | 0.00 | 0.54 | | 0.27 | 0.10 | 0.37 | 0.17 | |
| | 4/26 | 0.63 | 0.00 | 0.00 | 0.63 | | 0.67 | 0.12 | 0.79 | —0.16 | |
| | Average | | | | 0.59 | 0.029 | | | | 0.01 | 0.000 |
| III | G.E. | | | | | | | | | | |
| | 4/21 | 0.48 | 0.01 | 0.00 | 0.49 | | 0.32 | 0.09 | 0.41 | 0.08 | |
| | 4/26 | 0.52 | 0.01 | 0.00 | 0.53 | | 0.48 | 0.05 | 0.53 | 0.00 | |
| | Average | | | | 0.51 | 0.029 | | | | 0.04 | 0.002 |
| III | J.E. | | | | | | | | | | |
| | 4/21 | 0.56 | 0.01 | 0.00 | 0.57 | | 0.28 | 0.12 | 0.40 | 0.17 | |
| | 4/26 | 0.60 | 0.01 | 0.00 | 0.61 | | 0.45 | 0.08 | 0.53 | 0.08 | |
| | Average | | | | 0.59 | 0.029 | | | | 0.12 | 0.005 |
| III | J.E. | | | | | | | | | | |
| | 5/12 | 0.68 | 0.02 | 0.01 | 0.69 | | 0.63 | 0.07 | 0.70 | —0.01 | |
| | 5/17 | 0.58 | 0.02 | 0.01 | 0.59 | | 0.54 | 0.07 | 0.61 | —0.02 | |
| | Average | | | | 0.64 | 0.030 | | | | —0.02 | —0.001 |

[1] Slight regurgitation not analyzed.

[2] Estimated values.

their spectra with those produced by solutions of known concentration.

Neither the fecal nor the urinary excretion of aluminum bore any relation to the amount of aluminum ingested. Some aluminum was present in all the urines, although the greater proportion of the ingested aluminum appeared in the feces.

Fifty per cent of the balances were positive and 50% were negative. Sixty per cent of all of these fell within the $\pm 10\%$ error as determined for the method used in this study. The amount of aluminum retained in the remaining 40% of the balances suggested that aluminum is not an essential constituent of the diets of preschool age children.

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